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Inflammation-Induced Plasticity of Micturition Reflex Pathways

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INFLAMMATION-INDUCED PLASTICITY OF MICTURITION REFLEX
PATHWAYS

A Dissertation Presented

by

Lauren Arms

to

The Faculty of the Graduate College

of

The University of Vermont


In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Neuroscience

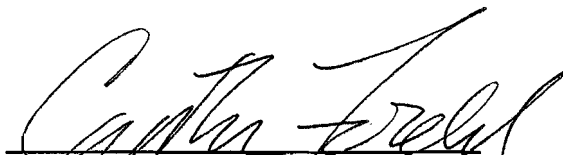
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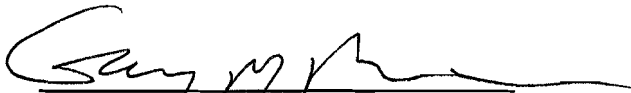
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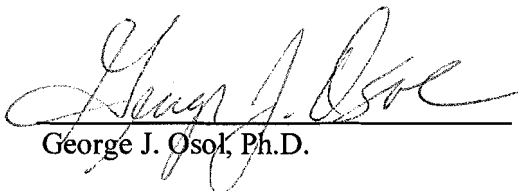
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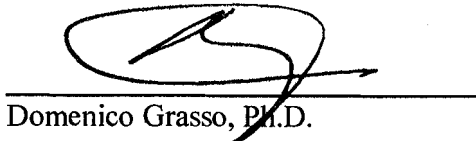

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Abstract

Although a seemingly basic and simple behavior, micturition necessitates precise integration and coordination of multiple divisions of the nervous system: visceral sensory, somatic motor, sympathetic, parasympathetic, as well as voluntary control from higher brain/ brainstem centers. When coordination of this circuitry falters, the consequences can be devastating and include severely decreased quality of life and substantial economic burden. This dissertation project investigates the potential role(s) of inflammatory mediators in bladder sensory physiology with the long term goal of elucidating potential targets for intervention. The overall hypothesis is that inflammatory-induced changes in the urinary bladder or afferent projections ultimately lead to dysfunctional micturition symptoms. Using a rodent model of cyclophosphamide (CYP)-induced bladder inflammation, we examined the expression and function of the chemokine/ receptor pair, CXCL12/CXCR4, and the activated (phosphorylated) form of ubiquitous signaling molecule, AKT using a multidisciplinary approach that includes: immunohistochemistry, protein and transcript quantification techniques, and *in vivo* bladder physiology studies combined with pharmacological tools.

Peripheral chemokine levels are elevated in patients with various chronic pelvic inflammatory/ pain syndromes including interstitial cystitis/bladder pain syndrome (IC/BPS) and are implicated in numerous inflammatory and mechanical pain models in rodents. However studies had not previously shown a direct functional role for chemokine signaling in micturition. We hypothesized that CXCL12 and CXCR4 would increase in the urinary bladder with CYP-induced bladder inflammation and that CXCR4 receptor blockade with AMD3100 would reduce CYP-induced bladder hyperreflexia. ELISA, immunohistochemical and qRT-PCR experiments demonstrate duration-dependent increases in CXCL12 and CXCR4 protein and transcript expression in specific tissue compartments of the urinary bladder, mainly the urothelium. *In vivo* studies provide evidence of a role for chemokine signaling in the mediation of micturition function. Intravesical infusion with AMD3100, a CXCR4 receptor antagonist, significantly reduced CYP-induced bladder hyperreflexia as evidenced by increased bladder capacity, intercontraction interval and decreased voiding frequency.

AKT is a putative cellular survival signal, however, recent studies also implicate the signaling molecule in the induction and maintenance of pain processes, development of long term plasticity (e.g. LTP and central sensitization) and visceral inflammation. Functional studies addressing the contribution of pAKT in micturition have not been performed. We hypothesized that increasing pAKT levels would contribute to CYP-induced bladder hyperreflexia. Western blot and immunohistochemical studies demonstrated that phosphorylation of AKT increases in the whole urinary bladder with CYP-induced bladder inflammation in a tissue compartment- and time-dependent manner. Intravesical infusion with inhibitors of AKT phosphorylation, AKT Inhibitor IV and deguelin, significantly improved symptoms of CYP-induced bladder hyperreflexia suggesting a functional role for pAKT in bladder physiology.

These studies demonstrate the functional capacity of inflammatory mediators and inflammatory associated signaling pathways in micturition reflex pathways. Chemokine signaling via the CXCR4 receptor and upstream activators of AKT may provide therapeutic targets with respect to inflammatory-induced bladder sensory physiology dysfunction.

Citations

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Dedication

For my mother, Susan Arms.
She is the epitome of strength and determination; she is my inspiration.

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First and foremost I thank my parents, Susan and Roger Arms, for the solid foundation they have worked tirelessly to create and maintain in all aspects of my life. Their encouragement throughout the years has enabled me to believe that I can achieve anything. I owe the success of my academic career and happiness in life to their unyielding and selfless support.

Graduate school has been an incredible learning and growing experience. I am immensely thankful to have worked with my advisor, Dr. Margaret Vizzard, who dedicated so much of her valuable time to me and my project. While working with Margaret, I gained invaluable skills that will help me undoubtedly throughout my career and personal life. Her unwavering kind and positive demeanor in the face of all situations, combined with her professional and personal ambitions make her an exceptional role model and a cultivating mentor. I would also like to thank members of the Vizzard lab, Susan Malley, Abbey Peterson, Kimberly Corrow and Dr. Beatrice Girard for all they have taught me, their willingness to always lend a helping hand, and their sincere friendship.

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I extend a special thank you to Andre Zdunczyk for his continued love, support and devotion. He finds a way to keep my feet on positive ground during all the ups and downs experienced while chasing my dreams.

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Abbreviations

CGRP: calcitonin gene-related peptide

CNS: central nervous system

COX-2: cyclooxygenase-2

DCM: dorsal commissure

DH: dorsal horn

EAA: excitatory amino acid

GPCR: g protein-coupled receptor

HIV: human immunodeficiency virus

IC/BPS: Interstitial cystitis/ bladder pain syndrome

IL: interleukin

IML: intermediolateral grey matter

iNOS: inducible nitric oxide synthase

LCP: lateral collateral pathway

LUT: lower urinary tract infection

MPG: major pelvic ganglion

MPO: myeloperoxidase

MS: multiple sclerosis

NSAIDs: non-steroidal anti-inflammatory drugs

PACAP: pituitary adenylate cyclase activating peptide

PG: prostaglandin

PMC: pontine micturition center

PD: Parkinson's disease

SCI: spinal cord injury

SDH: superficial dorsal horn

SP: substance P

SPN: sacral parasympathetic nucleus

TNF: tumor necrosis factor

Trk: tyrosine kinase

VIP: vasoactive intestinal peptide

Chapter 1: Comprehensive Literature Review

I. Introduction

Micturition is a fundamental and necessary human behavior. The essential role of the bladder is to store incoming urine from the kidneys and to execute periodic elimination. Although bladder function appears relatively simple, micturition necessitates precise integration and coordination of multiple divisions of the nervous system: sympathetic, parasympathetic and somatic, as well as voluntary control from higher brain/ brainstem centers. When changes in neuronal functions related to or within this circuitry occur, as in pathologic states (e.g. bladder outlet obstruction, overactive bladder, spinal cord injury, Parkinson's Disease, multiple sclerosis or Interstitial Cystitis/ Bladder Pain Syndrome) consequences can be devastating and include severely decreased quality of life and substantial economic burden. Injury or disease within micturition reflex circuitry can result in bladder hyperreflexia, hyporeflexia, incontinence, irritative voiding and associated pain. Little is known about the mechanisms underlying bladder dysfunction and therefore research investigating potential mechanisms and mediators is essential.

One type of bladder dysfunction is Interstitial Cystitis/Bladder Pain Syndrome (IC/BPS), a chronic syndrome of unknown etiology with symptoms of urgency, frequency and nocturia; additionally, patients experience pain that is localized beyond the bladder in suprapubic and pelvic regions. Theories as to the origin of IC/BPS include mast cell degeneration, infection, urothelial deficiency, autoimmune disorder, toxic urinary components and neurogenic causes, however no consensus has been reached.

Although each theory argues a different primary insult, common to each theory is an inflammatory component, either as a direct effector (e.g. mast cell degeneration, autoimmune disorder) or as a secondary result (e.g. urothelial deficiency, infection). Additionally, inflammatory cells and mediators are detected in the bladders and urine of patients with IC/BPS. Numerous inflammatory mediators exhibit functional roles during bladder inflammation in rodents.

Inflammation, especially chronic inflammation, may induce plasticity within the urinary bladder and/or its central and peripheral innervation leading to chronic bladder dysfunction. Inflammatory mediators may contribute to bladder dysfunction via sensitization of second order bladder afferents in the spinal cord or primary afferents. In the case of primary afferents, sensitization may occur either directly or indirectly via urothelium (the epithelial lining of the bladder) to neuron communication. In any case, a sensitized afferent limb of the micturition reflex pathway may ultimately lead to hyperalgesia and allodynia that, in terms of bladder function, would translate into heightened bladder sensation, increased voiding frequency and/or pain. The overall goal of this thesis dissertation is to further characterize the role of inflammation in bladder dysfunction. Specifically, I examine expression and functional role(s) of a chemokine/receptor pair, CXCL12/CXCR4, and a signaling molecule involved in inflammatory signaling, AKT, in the urinary bladder with inflammation.

II. Anatomy and Function of the Lower Urinary Tract

A. Anatomy of the Lower Urinary Tract

Micturition necessitates precise integration and coordination of multiple divisions of the nervous system. Lending to its complexity, the lower urinary tract (LUT), composed of the bladder, urethra and internal and external urethral sphincters, is composed of both striated and smooth muscle, and therefore under both voluntary (somatic) and involuntary (autonomic) influence (Figure 1) (Andersson and Arner, 2004). Based on regional differences in adrenergic innervation, the bladder is divided into two main parts: the body and the base (Elbadawi, 1996). Sympathetic adrenergic innervation is more dense in the base region compared to the body (Klück, 1980; Elbadawi, 1996). The base occupies the inferior portion of the urinary bladder (i.e. below the ureteral orifices) and includes the trigone, urethrovesical junction (bladder neck) and anterior bladder wall. As the name suggests, the trigone is the triangle-shaped area bounded by the ureteral orifices and the bladder neck along the posterior surface of the bladder wall (Elbadawi, 1996).

The urinary bladder wall has three layers: a urothelium on the luminal surface, a lamina propria just deep to the urothelium (a.k.a. the suburothelium), and an outer smooth muscle layer, named the detrusor (Andersson and Arner, 2004; Figure 1). The urothelium is composed of at least three layers: an apical layer on the luminal surface, an intermediate layer (multiple intermediate layers are present in human and other species) and a basal layer attached to a basement membrane. The highly specialized apical cells, also termed umbrella cells, are large, hexagonal shaped cells and have structural

characteristics that aid in the defense against toxic urine components (e.g. potassium) or bacterial adherence and infection. Sulfated polysaccharides or glycosaminoglycans (GAGs) are large, negatively charged molecules that line the entire apical surface of the urothelium (Parsons, 2007). Due to their negative charge, GAGs trap water molecules and solutes and thus create a mucous layer with anti-adherence properties along the luminal lining of the bladder (Parsons, 2007). Surface expression of specialized membrane lipids, together with dense networks of uroplakin proteins, restrict the flow of toxic urinary components into the urothelium or underlying bladder tissue (e.g. suburothelial plexus of afferent nerve fibers) (Lewis, 2000; Birder, 2006). Uroplakins are urothelium- specific crystalline proteins that assemble into hexagonal plaques and constitute the majority of apical urothelium protein content (Staehelin et al., 1972; Birder, 2006). Additionally, transmembrane proteins assist in cell-cell adhesion and tight junctions restrict the flow of water and ions between cells (Claude and Goodenough, 1973; Birder, 2006).

Increased barrier permeability is thought to contribute to symptoms of bladder dysfunction in various bladder pathologies (Parsons et al., 1991; Hurst et al., 2007; Parsons, 2007). Urinary potassium levels are decreased in bladders from patients with IC/BPS, a chronic bladder inflammation syndrome, suggesting transurothelial absorption into the bladder and underlying structures (i.e. suburothelial nerve plexus, interstitial cells, detrusor smooth muscle) (Parsons et al., 2005). Treatment with heparin, an exogenous GAG, often relieves symptoms of IC/BPS probably by restoring the mucus layer and barrier function (Parsons et al., 2005; Parsons, 2007).

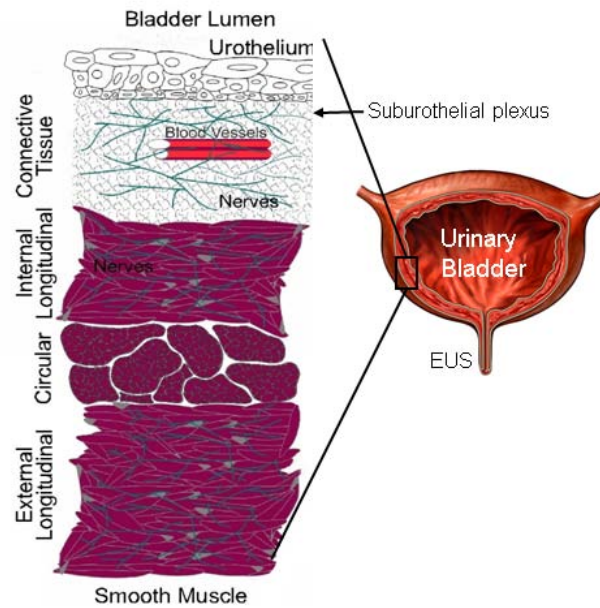


Figure 1. Anatomy of the lower urinary tract. The LUT is composed of the urinary bladder, internal and external urethral sphincters and the urethra. The bladder consists of 3 layers: a urothelium on the luminal surface, a lamina propria just deep to the urothelium that contains a suburothelial plexus of nerve fibers and vasculature, and an outer muscle layer, named the detrusor that contains both longitudinal and circular smooth muscle. The external urethral sphincter is composed of striated muscle and encircles the urethra creating a diaphragm that allows for the voluntary control of urine flow. EUS, external urethral sphincter.

Deep to the urothelium is a lamina propria termed the suburothelium. Within the suburothelium are dense neuronal and vascular networks (Figure 1). Sensory fibers within the lamina propria are most dense in the neck region of the bladder. Populations of interstitial cells (a.k.a. myofibroblasts) also reside in the suburothelium. Although the role of interstitial cells is uncertain, some suggest that they have a pace-making (McCloskey and Gurney, 2002) or intermediary role between neurons and detrusor smooth muscle cells (Sui et al., 2002; Andersson and Arner, 2004). Expression of functional gap junctions supports the plausibility of this hypothesis (Sui et al., 2002).

The detrusor muscle is composed of longitudinal and circular smooth muscle fibers. Muscle cells are encased into bundles with collagen-rich connective tissue. The bundles of smooth muscle cells weave amongst each other and do not follow a discernable pattern (Murakumo et al., 1995; Andersson and Arner, 2004). Gap junctions between cells allow for rapid communication and for coordinated contraction of the entire bladder wall.

The urethra and associated sphincters are the remaining components of the LUT. The urethra contains smooth muscle that is structurally and functionally different than bladder smooth muscle. The internal urethral sphincter, a smooth muscle sphincter, is located just inferior to the bladder at the urethrovesical junction (Elbadawi, 1996; Fowler et al., 2008). In contrast, the external urethral sphincter, located inferiorly to the prostate in males or at the inferior end of the urethra in females, is under voluntary control as it is a striated muscle sphincter (Elbadawi, 1996). The external urethral sphincter encircles the urethra creating a diaphragm that contributes to continence.

B. Neural Control of Micturition

The bladder is unique in that it requires constant central control. Proper bladder function is biphasic and includes a storage mode, under sympathetic control, and an elimination mode, under parasympathetic control. Local spinal reflexes, in conjunction with higher brain/brainstem centers, orchestrate the phasic or switch-like behavior in the bladder by via inhibition of pro-voiding centers during the storage mode and initiating the switch to elimination mode at the appropriate times (de Groat et al., 1982; de Groat et al., 1998). During storage mode sympathetic activation inhibits the detrusor muscle causing it to relax, and excites the bladder neck and urethra causing them to contract (Kingsley, 1996; Holstege, 2005; Fowler et al., 2008). The result is an elastic bladder able to accommodate incoming urine from the kidneys, as well as a constricted outflow to prevent involuntary loss of urine through the urethra. In addition, contraction of the external urinary sphincter via somatic motor output closes the urethra to urinary flow, and in combination with sympathetic output, allows for urinary continence (Kingsley, 1996; Holstege, 2005; Fowler et al., 2008). During elimination mode, parasympathetic activation sends excitatory signals to the detrusor causing it to contract, and thus creates enough pressure to void the bladder of urine entirely during micturition (Holstege et al., 1986; Blok and Holstege 1997; Sasaki, 2005); at the same time, both sympathetic and somatic motor input to the bladder, urethra and sphincter muscles must be turned off (Kingsley, 1996; Blok et al., 1997; Sie et al., 2001; Holstege, 2005; Fowler et al., 2008). Micturition centers in the brainstem receive afferent information in regard to filling (Griffiths et al., 2005; Kuipers, 2006). When a bladder threshold volume has been

reached, these centers initiate the change from storage to elimination mode (Holstege 1986; Block et al., 1997; Blok et al., 1998; Sasaki, 2005; Holstege, 2005; Fowler et al., 2008). However, through learned behavior, higher brain centers quell the micturition reflex until it is socially appropriate to void (Block et al., 1997; Blok et al., 1998; Griffiths et al., 2005). Therefore, complete and socially appropriate micturition requires the precise coordination of sympathetic, parasympathetic and somatic motor outflow, in conjunction with visceral sensory information and volitional control by the central nervous system.

In humans, all peripheral control for micturition originates from the lower thoracic to the sacral spinal cord. Specifically, sympathetic innervation originates from the intermediolateral grey matter (IML) of spinal segments T11-L2 (Holstege, 2005; Fowler et al., 2008). Preganglionic sympathetic fibers travel through the sympathetic chain to synapse in the inferior mesenteric ganglion or major pelvic ganglion (Fowler et al., 2008; de Groat and Yoshimura, 2010). Post-ganglionic sympathetic fibers travel in the hypogastric nerve and pass to both the bladder and urethra. In the detrusor smooth muscle, they elicit inhibitory effects by releasing norepinephrine onto β_3 adrenergic receptors thus causing the bladder to relax and allow for filling (de Groat and Suam, 1972; Fowler et al., 2008). In the urethra, however, postganglionic sympathetic fibers have an excitatory influence on urethral smooth muscle by releasing norepinephrine onto α_1 adrenergic receptors resulting in contraction (Flood, 1990; Fowler et al., 2008). Somatic motor outflow aids the sympathetic division in maintaining urinary continence by exciting the external urethral sphincter via release of acetylcholine onto nicotinic

receptors (Fowler et al., 2008). Because somatic motor innervation to the external urethral sphincter must synchronize with autonomic innervation of the LUT, the cell bodies of the motor neurons originate from a specialized nucleus, named Onuf's nucleus, in the anterior horn (lamina IX) (Schröder, 1981; Kingsley, 1996; Fowler et al., 2008) of sacral spinal cord segments S2-S4 (Fowler et al., 2008). The somatic motor axons pass along the pudendal nerve to the urinary sphincter.

Sensory information from the bladder and urethra associated with bladder pressure is carried via A- δ fibers in the hypogastric (bladder body and bladder neck), pudendal (urethra) and pelvic nerves (bladder body) (de Groat et al., 1982; Fowler et al., 2008). Filling continues until the bladder reaches near capacity. As the bladder approaches capacity, tension drastically increases, and stretch mechanoreceptors in the bladder wall elicit intense afferent firing (de Groat et al., 1982; Fowler et al., 2008). Cell bodies of these afferent fibers originate in the dorsal root ganglia (DRG) of spinal levels T11-L2 and S2-S4 (Holstege, 2005; Fowler et al., 2008). Central terminals of bladder primary afferents enter the spinal cord through Lissauer's tract and terminate on interneurons and spinal tract neurons in the superficial dorsal horn (SDH; lamina I), dorsal commissure (DCM; lamina X) and sacral parasympathetic nucleus (SPN; lamina V-VII) (Morgan et al., 1981; Kingsley, 1996; de Groat and Yoshimura, 2010). Ascending projections head rostrally to the periaqueductal gray matter (PAG) in the midbrain where bladder afferent information is integrated (Holstege, 2005; Fowler et al., 2008). Bladder interneurons may assist in local spinal cord reflexes (Birder et al., 1999) that maintain functional homeostasis including inhibition of parasympathetic pre-ganglionic neurons

during storage mode (de Groat and Yoshimura, 2010). This reflex is termed the guarding reflex and neurons involved in the communication between bladder primary afferents and parasympathetic motor output are located in the lateral collateral pathway (LCP) (Morgan et al., 1981; de Groat and Yoshimura, 2010).

The medial PAG receives afferent information regarding bladder filling and volitional control from spinal cord tract neurons or cortical/subcortical structures (e.g. prefrontal cortex, insular cortex and limbic system) (Holstege, 2005; Fowler et al., 2008). Connections with these areas are robustly inhibitory and reflect the importance of emotion and executive function involved in choosing an appropriate time and place to void (Griffiths et al., 2005). However, when environmental cues are favorable for micturition, and when sufficient bladder filling has occurred, cortical inhibitions are reduced and, together with volitional signals, cause the lateral PAG to activate projections to the pontine micturition center (PMC) located in the dorsolateral pontine tegmentum (Sasaki, 2005; Holstege, 2005). The PMC, particularly on the right side in humans, is the main excitatory center that initiates voiding (Blok et al., 1998; Komiyama et al., 1998; Holstege, 2005). Without endogenous or artificial activation of the PMC, micturition does not occur (Komiyama et al., 1998; Holstege, 2005). Axons projecting from the PMC travel in the lateral and dorsolateral funiculus along the spinal cord before synapsing with bladder associated interneurons and preganglionic neurons in the thoracolumbar and sacral segments (Holstege, 2005). PMC projections directly excite parasympathetic neurons in the SPN in the sacral spinal cord (human S2-S4) to initiate detrusor smooth muscle contraction while other projections assist in sympathetic and

somatic motor inhibition (Holstege et al., 1986; Blok and Holstege, 1997; Blok et al., 1997; Sie et al., 2001; Fowler et al., 2008).

Pre-ganglionic parasympathetic axons travel in pelvic nerves and head to and synapse in the pelvic ganglia. Post-ganglionic parasympathetics head to the detrusor smooth muscle where they will assert an excitatory influence by releasing acetylcholine onto muscarinic-3 (m3) receptors and, to a lesser extent, ATP onto P2X₂, P2X₃, P2X_{2/3} purinergic receptors, thus causing the bladder to contract (Carpenter, 1977; Burnstock et al., 1978; Dean and Dowie 1978; Holt et al., 1985; Fowler et al., 2008). For efficient urinary expulsion, the PMC must also inhibit sympathetic and somatic motor neurons in order to relax the bladder neck and external urinary sphincter muscle. To do so, PMC neurons synapse on γ -aminobutyric acid (GABA)-ergic and glycinergic interneurons in the spinal cord that in turn inhibit neurons in the sympathetic and Onuf's nuclei (Blok et al., 1997; Sie et al., 2001; Holstege, 2005). It has also been suggested that nitric oxide released from parasympathetic post-ganglionics in the urethra causes relaxation of the urethral smooth muscle (Dokita et al., 1991; Persson and Andersson 1992; Persson and Andersson, 1994). PMC activation results in bladder contraction and sphincter relaxation, thus allowing for complete bladder emptying (Figure 2).

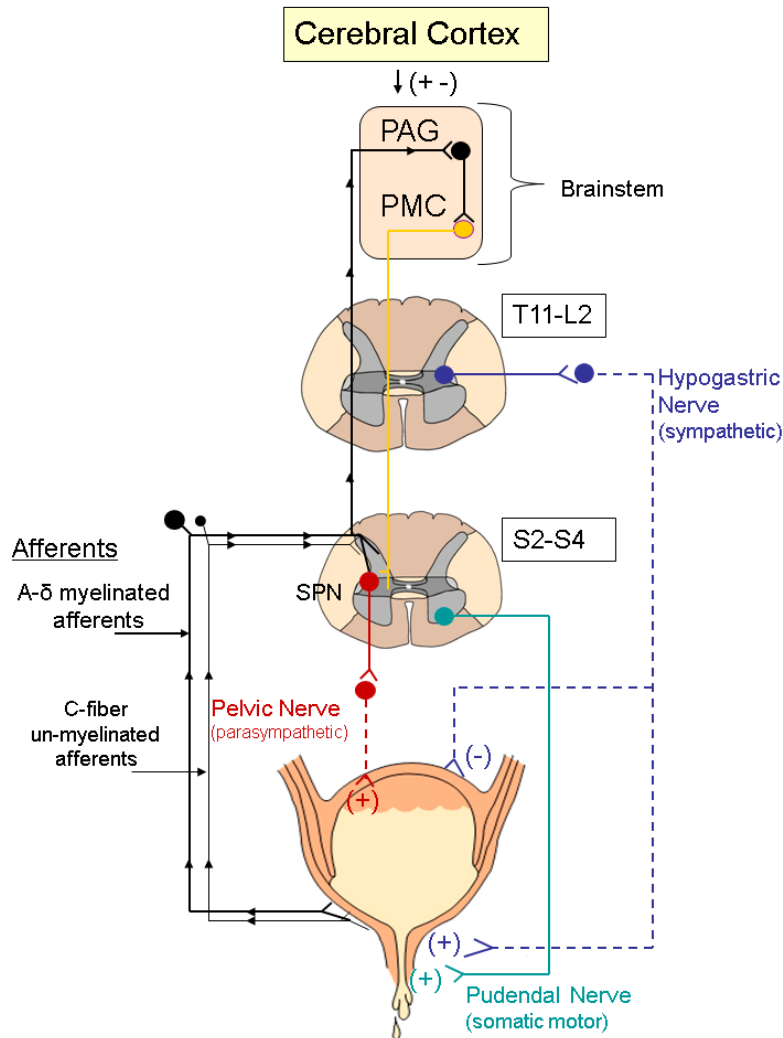


Figure 2. Neural control of micturition. Sympathetic innervation of the urinary bladder is responsible for relaxation of the bladder and contraction of bladder neck during filling. Pre-ganglionic sympathetic cell bodies originate from T11-L2 spinal cord levels in humans and travel in the hypogastric nerve. Somatic motor control originates from S2-S4 spinal cord levels, travels in the pudendal nerve and contracts the external urethral sphincter during filling to maintain continence. Parasympathetic cell bodies are also located in spinal cord levels S2-S4. Parasympathetic fibers travel in the pelvic nerve and are responsible for the intense bladder contraction during voiding. Bladder afferents ascend to higher brain/brainstem centers such as the PAG, the main center responsible for receiving and integrating bladder afferent information. The PMC initiates the switch from storage to elimination mode. PAG, periaqueductal gray; PMC, pontine micturition center.

C. Awakening of c-fiber Afferents

A second type of afferent neuron, the unmyelinated, capsaicin- sensitive c-fiber afferent, also innervates the urinary bladder. Although c-fiber afferents comprise approximately 70% of bladder afferents, c-fibers are quiescent and play no role during physiologic bladder filling and emptying (Fowler et al., 2008). Bladder c-fibers do respond to noxious stimuli such as cooling, toxic urinary components, pathologically high intravesical pressure and inflammation (Fall et al., 1990; Häbler et al., 1990; Jänig and Koltenburg, 1990; Chai et al., 1998; Yoshimura et al., 2007; de Groat, 2010; Kanai and Andersson, 2010). C-fiber activation is thought to contribute to plasticity of micturition reflex pathways. For example, suprasacral spinal cord injury causes loss of brainstem and cortical input to micturition reflex pathways and this involves “awakening” of c-fiber afferents. The micturition reflex partially recovers by adapting from a spinobulbospinal pathway into a local spinal cord reflex. Changes in the reflex pathways include activation of primary afferent c-fibers during bladder filling. Activated c-fiber-induced sensitization of the afferent limb of the micturition reflex is thought to mediate symptoms related to bladder dysfunction and pain in various types of bladder pathologies including IC/BPS, suprasacral spinal cord injury (de Groat et al., 1990; Cheng et al., 1999; de Groat and Yoshimura, 2006; Fowler et al., 2008) and bladder outlet obstruction (Häbler et al., 1990; Chai et al., 1998).

D. Cystometry: Evaluation of Bladder Function in Humans and Animals

Cystometry is a technique commonly used to evaluate bladder function in both experimental animals and humans. Cystometry entails continuous infusion of fluid (e.g.

saline) into the bladder while measuring bladder reflex parameters. Typical measurements recorded during cystometry include filling pressure (the pressure during the filling phase of the micturition cycle), threshold pressure (pressure at the beginning of the void event), peak micturition pressure (the highest pressure reached during voiding), intercontraction interval (the time lapsed between the end of one void event and the end of the next), void volume, bladder capacity and presence/ frequency of non-voiding contractions (Maggi et al., 1986).

In experimental rodent models of cystometry a urinary catheter is secured at one end in the dome, or most caudal region, of the bladder and the other end runs subcutaneously through the thorax before being externalized at the base of the neck. This externalization allows for easy access during saline and/or drug infusions during conscious cystometry. Both conscious and anesthetized cystometry experimental models are utilized in the current literature; however, major urodynamic differences exist between the two models. Importantly, bladder capacity is significantly increased under anesthesia (Ghoniem et al., 1996; Yokoyama et al., 1997). General anesthesia may block glutamate transmission in the brain thus affecting the micturition reflex (Ghoniem et al., 1996; Yokoyama et al., 1997). Therefore, conscious cystometry more closely resembles physiologic bladder functions. However, the surgical implantation of the tubing into the bladder may affect bladder sensation or detrusor contractility, therefore a recovery period of at least several days is allotted before urodynamic testing.

Urodynamic parameters are attributed to either the efferent or afferent limb of micturition pathways (Maggi et al., 1986). Experiments involving blockade of nicotinic

signaling in micturition reflex pathways should ablate the efferent control of the urinary bladder. In the presence of nicotinic blockade, peak amplitude of micturition decreased and voiding efficiency became problematic, thus leaving residual urine in the bladder. Presumably due to this increased residual urine, resting and threshold pressures increased, suggesting that neuronal pathways regulating resting and threshold pressures remained intact. Additionally, as time progressed, intercontraction interval decreased in response to additive residual volume over repeated cycles of saline instillation suggesting that bladder capacity had not changed with nicotinic blockade (Maggi et al., 1986). Nicotinic blockade impaired the ability of the bladder to evacuate urine entirely, but not the ability of the bladder to detect intraluminal volumes, therefore, peak amplitude of micturition and void volume are under efferent control while threshold pressure, resting pressure, intercontraction interval and bladder capacity are associated with the afferent limb of the reflex pathway (Maggi et al., 1986).

The presence of non-voiding contractions (i.e. significant rises in bladder filling pressure that does not elicit expulsion of urine) is atypical in cystometric recordings from healthy humans or experimental animals. However, various forms of bladder pathology (e.g. spinal cord injury, bladder outlet obstruction and bladder inflammation) are associated with the emergence of non-voiding contractions. Neither the function nor the origin of these contractions is fully understood. Both myogenic and neurogenic mechanisms have been proposed (Brading et al., 1997; Herrera et al., 2003; Comiter et al., 2010). Experimental manipulations show the ability of isolated bladder smooth muscle strips to increase the number of spontaneous activity or phasic contractions in

response to stretch in the absence of urothelium or intact neuronal innervation, and therefore imply a myogenic mechanism for the induction of non-voiding contractions (Brading, 1997; Herrera et al., 2003). Alternatively, neurogenic mechanisms may also contribute to the presence of non-voiding contractions either directly (e.g., by increasing spontaneous firing) or indirectly by facilitating functional changes in detrusor smooth muscle myocytes (Brading et al., 1997; Comiter et al., 2010). Comiter et al., (2010) demonstrate that sacral nerve stimulation decreases the number of non-voiding contractions observed in a model of bladder outlet obstruction without affecting detrusor contractility. Interestingly, sacral nerve stimulation also attenuates structural changes such as hypertrophy and fibrosis observed in the bladder following outlet obstruction (Comiter et al., 2010). Therefore, the genesis of non-voiding contractions may reflect a combination of neuronal and muscular changes that could reciprocally influence each other to produce phenotypic changes observed in bladder pathology. Other explanations include changes in the sensory machinery of the urothelium that may alter the bladder's response to distension, possibly via communication to the bladder afferents, interstitial cells or detrusor smooth muscle located deep to the urothelium (Herrera et al., 2003).

II. Micturition in Injury or Disease

Bladder function relies entirely on central nervous system (CNS) control and, therefore, is vulnerable to injury or disease. When orchestration of the circuitry fails, the effects can range from total incontinence to complete retention of urine, but it almost always results in a decreased quality of life. Symptoms depend on the location of lesion or nature of synaptic rearrangement. Brain lesions usually lead to a hyperreflexic bladder

due to its strong inhibitory influence on micturition behavior. Changes in the spinal cord grey or white matter can have different effects such as bladder hypo- or hyperreflexia and bladder associated pain.

A. Spinal Cord Injury

Spinal cord injury presents a multitude of challenges, and loss of continence can be one of the toughest emotional hurdles with which to cope. Spinal cord injury triggers an immediate and acute shock response of the nervous system followed by a period of reorganization and partial recovery (Yoshimura, 1999). For injuries at or above the lumbosacral spinal cord, the bladder becomes completely flaccid and areflexic causing total retention; however, after a recovery period, synaptic changes in the peripheral innervation of the LUT allow the detrusor to regain slowly its contractile properties (de Groat et al., 1982; Yoshimura, 1999). Despite a partial recovery of the micturition reflex, synaptic rearrangements and loss of precise coupling between somatic relaxation of the external urethral sphincter muscle with parasympathetic contraction of the detrusor smooth muscle causes detrusor-sphincter dyssynergia; in other words, as detrusor contraction attempts to expel the bladder in full, the urinary sphincter rhythmically tightens, thus retaining residual urine within the bladder (Yoshimura, 1999; Fowler et al., 2008). Incomplete or failed emptying necessitates repeated catheterizations that increases risk for urinary tract infections. This situation is particularly dangerous because urinary tract infections can trigger the potentially fatal, autonomic dysreflexia syndrome in spinal cord patients (Kingsley, 1996).

B. Multiple Sclerosis

Multiple sclerosis (MS), a demyelinating disease of the central nervous system, also may interrupt normal micturition pathways. Depending on the site of lesion, urinary symptoms exhibited by many MS patients include detrusor hyperreflexia, hyporeflexia and/or detrusor-sphincter-dyssynergia (Haensch and Jörg, 2006). Functionally, these symptoms relate to sensations of urgency, the need for frequent elimination and/or incomplete bladder emptying. Lesions to the cortex most likely lead to a hyperreflexic bladder due to its main inhibitory influence over the micturition reflex. Lesions in the pons or suprasacral spinal cord can lead to detrusor hyporeflexia or detrusor-sphincter-dyssynergia because they disrupt the pathways responsible for precise coordination of all divisions of the nervous system during the switch from the storage phase to the emptying phase (Betts et al., 1993; Araki et al., 2003; Haensch and Jörg, 2006).

C. Parkinson's Disease

Parkinson's disease (PD), a progressive degenerative disorder of the CNS, also affects autonomic functions including micturition. PD results from a loss of dopaminergic neurons in the substantia nigra. Normally, these dopaminergic neurons facilitate an overall inhibitory effect on micturition. With a disrupted dopaminergic circuitry, the PMC fails to receive adequate inhibition resulting in a hyperreflexic bladder (Kitta et al., 2008). In addition, evidence suggests that under normal circumstances, the D1 dopaminergic pathway may send collateral inhibitory GABAergic collaterals to the PMC thus further increasing its role in the storage phase of micturition (Kitta et al.,

2008). Not surprisingly, 45-93% of PD patients suffer from overactive bladders (Kitta et al., 2008).

D. Interstitial Cystitis/ Painful Bladder Syndrome (IC/BPS)

While much is unknown about the micturition defects observed in MS and PD patients, still other disease mechanisms remain even more elusive. Interstitial Cystitis/ Bladder Pain Syndrome (IC/BPS) is a chronic bladder syndrome with presenting symptoms of urgency, frequency, nocturia, suprapubic and pelvic pain. IC/BPS patients have a lower threshold for sensing bladder volume and often feel pain at normal bladder volumes which leads to decreased bladder capacities (FitzGerald et al., 2005; Nazif et al., 2007). Additionally, pain localization extends beyond the region of the bladder. In patients with IC/BPS, bladder fullness causes discomfort at suprapubic, urethral and vulvar sites (Fitzgerald et al., 2005; Warren et al., 2008). Histopathological findings from IC/BPS biopsies reveal edema and vasodilatation in the bladder (Nazif et al., 2007). While some theories exist as to the underlying mechanisms of this syndrome, the etiology remains largely unclear.

Basic research for IC/BPS is crucial because an estimated 1.3-12 million Americans are diagnosed with the syndrome (Interstitial Cystitis Association, 2010. Home Page. (n.d.) Retrieved from www.ichelp.org; National Kidney and Urologic Disease Information Clearinghouse, 2010. *Interstitial Cystitis/Painful Bladder Syndrome*. (n.d.) Retrieved from <http://kidney.niddk.nih.gov/kudiseases/pubs/interstitialcystitis/index.htm>). Because the symptoms of IC/BPS necessitate frequent trips to the bathroom during both the day and nighttime, patients with IC/BPS are often unable to

work or may even suffer from depression. As a result, IC/BPS costs to the government include both medical care and loss of productivity which an estimate from 1987 was approximately \$428 million (Association of Reproductive Health Professionals, 2010. Publications and Resources. *Screening, Treatment and Management of IC/PBS*. May 2008. Retrieved from www.arhp.org/Publications-and-Resources/Clinical-Proceedings/Screening-Treatment-and-Management-of-ICPBS/Epidemiology).

For unknown reasons, women comprise the majority of those diagnosed with IC/BPS, accounting for nearly 90%. However, rates among men are increasing due to IC/BPS awareness and the rectifications of incorrect prostatitis diagnoses. To further complicate matters, IC/BPS is probably a multiple disease syndrome as biopsy findings, pathologic urinary markers and history of urinary tract infections vary from patient to patient (Erikson, 1999).

Life stress has been associated with onset and severity of IC/BPS symptoms (Rothrock et al., 2001). Episodes of urgency, pain and nocturia correlate with self reported stress and these associations become stronger with increasing severity of symptoms (Rothrock et al., 2001). The lack of understanding of the pathology of IC/BPS combined with the heterogeneity of symptoms and patient population present enormous challenges when trying to establish effective treatments. The only FDA-approved intravesical treatment for IC/BPS is instillation of dimethyl sulfoxide (DMSO). DMSO moderately reduces IC/BPS symptoms and is thought to act via anti-inflammatory, muscle relaxant and/or neuronal desensitization mechanisms (Birder et al., 1997; Wehbe et al., 2010). Other intravesical treatments include instillation of local anesthetics (e.g.

lidocaine) or pentosan polysulfate sodium (PPS). PPS is a polysaccharide that is thought to restore the GAG layer and barrier function to the mucosal layer of the bladder. PPS is also the only approved oral medication for the treatment of IC/BPS (Wehbe et al., 2010). Other oral remedies include dietary modification, antihistamines, muscle relaxants and herbs or other naturally occurring compounds with antioxidizing and anti-inflammatory properties such as quercetin, a bioflavonoid found in red wine, green tea, seeds, citrus fruits and onions (Whitmore, 2002; Wehbe et al., 2010).

Two alternative types of treatment, sacral nerve modulation and myofascial trigger point therapy, attempt to reduce heightened tension of the pelvic floor muscles that is often present in patients with IC/BPS. Some researchers argue that pelvic floor dysfunction precedes or may cause dysfunctional urinary bladder symptoms. Sources of pelvic floor dysfunction include improper exercise or movement, trauma during sexual activity, surgically-induced hypersensitivity or viral infection (Weiss, 2001). Intense sensory afferent firing from somatic sensory nerves innervating the pelvic floor may activate convergent bladder afferent neurons in the sacral spinal cord (Weiss, 2001). In fact, a recent controlled, randomized study evaluating the efficacy of myofascial trigger point therapy to reduce perceived severity of pain and urinary symptoms demonstrates, for the first time in 10 years, statistically significant positive outcomes for the treatment of IC/BPS (Payne et al., 2010). However the lack of positive results from previous studies probably reflects the heterogeneity of the IC/BPS population. Future research might reveal that the IC/BPS diagnosis encompasses multiple cohorts of urinary bladder dysfunction. The origin and pathogenesis in each may differ and therefore explain the

difficulties in establishing an effective universal treatment for IC/BPS. Additional theories as to the etiology and pathogenesis of IC/ BPS include infection, autoimmune disorder, toxic urinary components, deficiency in bladder wall lining and neurogenic causes.

III. Inflammation and IC/BPS

Despite fundamental differences in the primary cause of IC/BPS symptoms, the activation of inflammatory mediators, either as a direct insult or as a secondary result, is a common theme in each proposed mechanism. The majority of biopsies from IC/BPS patients exhibit infiltration of inflammatory cells or mediators, such as lymphocytes, T cells, B cells, mast cells, interleukin-6 and interleukin-2 (Erickson et al., 1997) suggesting that inflammatory mediators may contribute to dysfunctional bladder symptoms (Batler et al., 2002; Yoshimura et al., 2002; Sant et al., 2007). Rodent studies utilizing chemically-induced cystitis and clinical trials involving patients with IC/BPS demonstrate that treatment with an immunoregulator (suplatast tosilate, IPD-1151T) that suppresses cytokine production, immunoglobulin E antibody formation and mast cell activation, can increase bladder capacity and reduce pain scores (Ueda et al., 2000; Yoshimura et al., 2002). Potential mediators of bladder inflammation are numerous and include cytokines, chemokines, neuropeptides, neuroactive compounds and growth factors.

A. Nerve Growth Factor

Nerve growth factor (NGF), a potent neurotrophin, is also an important mediator of somatic and visceral pain and inflammation. NGF is elevated in the urine and

urothelium of patients with IC/BPS and therefore research has focused on the contributions of NGF/ receptor signaling to symptoms associated with IC/BPS such as bladder hyperreflexia and referred somatic pain (Okragly et al., 1999; Lowe et al., 2007). Chemically-induced bladder inflammation in rodent models has demonstrated changes of NGF and its associate receptors, TrkA, TrkB and p75^{NTR} in the urinary bladder, bladder primary afferents and major pelvic ganglia (Vizzard, 2000; Qiao and Vizzard, 2002; Murray et al., 2004; Klinger et al., 2008). Addition and subtraction experiments have demonstrated functional roles for NGF in micturition reflex pathways. For example, direct application of NGF to the detrusor smooth muscle, lumen of the bladder, lumbosacral intrathecal space or adenovirus-induced NGF expression in the detrusor smooth muscle causes sensitization of bladder afferents and bladder hyperreflexia as evidenced by decreased bladder capacity and intercontraction intervals (Dmitrieva et al., 1997; Chuang et al., 2001; Lamb et al., 2004; Yoshimura et al., 2006; Zvara and Vizzard, 2007). Chronic application of NGF to the detrusor smooth muscle induces expressional increases and spatial alterations of both Fos protein, a marker of cellular activation, and calcitonin gene-related peptide (CGRP), a protein known to be involved in noxious sensation in the lumbosacral spinal cord (Zvara and Vizzard, 2007). Conversely, agents that decrease NGF levels can attenuate its hyperreflexic effects (Dmitrieva et al. 1997; Hu et al., 2005). Most interestingly, transgenic mice overexpressing NGF under the urothelium-specific uroplakin II promoter exhibit symptoms similar to those observed in IC/BPS: hyperinnervation of unmyelinated and myelinated sensory fibers as well as sympathetic nerves in the suburothelial plexus, increased urinary bladder reflex activity

and referred somatic hypersensitivity in the pelvic region (Schnegelsberg et al., 2010). These data strongly implicate a role for NGF signaling in bladder sensory/physiology dysfunction.

B. *In vivo* Models of Urinary Bladder Inflammation

The etiology of IC/BPS remains elusive; therefore, development of a model to study the underlying mechanisms produces challenges. Various models attempt to mimic the histopathological and functional changes affecting the urinary bladder observed in IC/BPS, but no one model encompasses all aspects of the syndrome; therefore, no single model is accepted universally as the standard to study IC/BPS.

B.1 Intravesical infusion of noxious chemicals

Some models of bladder inflammation involve intravesical infusion of noxious chemicals, such as acetone (Kato et al., 1990), acetic acid (Birder and de Groat, 1992; Yu and de Groat, 1998; Chuang et al., 2004), hydrochloric acid (Rivas et al., 1997; Wang et al., 2000; Cayan et al., 2003; Kirimoto et al., 2007), mustard oil and turpentine (McMahon and Abel, 1987) via transurethral catheterization. While the goal of such models is to induce inflammation, catheterization can induce tissue irritation and inflammation in rodents because of anatomical relationships and the small size of the urethra. Additionally, transurethral catheterization of chemical irritants increases the risk of introducing bacteria to the LUT, thus potentially causing urinary tract infection. Both potential side effects could confound results illustrating bladder hyperreflexia or increased suprapubic and pelvic pain. The toxic nature of the chemicals may cause tissue necrosis, a characteristic not found in biopsies of IC/BPS bladders (Kato et al., 1990).

Nevertheless, these agents induce histopathological and functional changes that appear similar to those observed in human IC/BPS (Rivas et al., 1997; Kirimoto et al., 2007). For example, hydrochloric acid causes edema in the lamina propria, infiltration of inflammatory cells as well as increased voiding frequency (Rivas et al., 1997; Kirimoto et al., 2007). Turpentine and mustard oil stimulate leukocyte infiltration and bladder hyperreflexia (McMahon and Abel, 1987). Although studies utilizing intravesical instillation of noxious chemicals have advanced knowledge in the IC/BPS field, short lasting effects of the method render translation of results for the human syndrome difficult. In humans, the syndrome is characterized by chronic, long lasting alterations in bladder activity and the tissues of the LUT; however, intravesical treatment with chemicals such as mustard oil and turpentine have induced bladder hyperreflexia that lasts only a relatively short 6 and 24 hours, respectively (McMahon and Abel, 1987).

B.2 Urothelial Damage: Protamine Sulfate

An interesting technique using protamine sulfate (PS) attempts to mimic urothelial barrier dysfunction observed in the bladders of IC/BPS patients. PS increases urothelial permeability allowing urinary components to diffuse into the bladder tissue (Tzan et al., 1993). Solutes such as potassium ions may depolarize bladder sensory neurons thus potentiating bladder overactivity. Chuang et al. (2003) showed that following intravesical infusion of PS, infusion of physiological saline solution into the bladder induces overactivity. While this technique offers insight into to the role of the urothelium, it lacks any inflammatory component. Since most IC/BPS bladder biopsies reveal some degree of inflammation (Rivas et al., 1997; Kirimoto et al., 2007), a model

that incorporates an inflammatory element is essential. Stein and colleagues (1996) followed PS treatment with endotoxin lipopolysaccharide (LPS) intravesical instillation. LPS is a well known model to stimulate visceral-specific inflammation (Stein et al., 1996). PS-LPS consecutive instillation caused urothelial breakdown and neutrophil and macrophage infiltration similar to histologic finding in patients with IC/BPS (Erickson et al., 1997). Although no functional studies were performed, this model may be a more accurate simulation of IC/BPS because it incorporates urothelial breakdown with an inflammatory component. Although models utilizing PS offer some insight to mechanisms underlying of IC/BPS, these models constrain hypotheses to focus on a dysfunctional urothelium as the cause of IC/BPS. While urothelial dysfunction is thought to contribute to symptoms of IC/BPS, other factors such as altered bladder sensory circuitry may also contribute to signs and symptoms of IC/BPS. Not only do PS models fail to incorporate other well-studied aspects of bladder dysfunction, but they also lack the ability to study the initiating factors of urothelial breakdown in IC/BPS bladders.

B.3 Naturally occurring Feline Interstitial Cystitis (FIC)

FIC is a naturally occurring model of IC/BPS. Using all inclusion and exclusion criteria applicable to humans, cats with FIC meet the National Institute of Health criteria for IC (Westropp and Buffington, 2002). Cats with FIC exhibit similar symptoms of urgency and frequency in the absence of bacterial infection (Lavelle et al., 2000). Histopathological changes in the bladders of cats with FIC are similar to those in humans with IC/BPS as they exhibit urothelial lesions and nonspecific inflammation (Lavelle et al., 2000). Additionally, as in humans, onset of the syndrome is spontaneous, may occur

at any age, and symptoms may enter a pattern of relapse and remission partially influenced by environmental stress (Westropp and Buffington, 2002). Despite many similarities between human IC/BPS and FIC, gender ratios of those afflicted in the two populations differ immensely. In the feline population, FIC occurs equally amongst males and females; however, in the human population, females account for 90% of IC/BPS cases. This disparity, along with other limitations associated with the model, deter many from using the FIC model to study IC/BPS. Because FIC is not definitively genetic and occurs spontaneously at any age, acquisition of sufficient numbers of animals to perform scientific investigations presents enormous challenges. Purchasing and animal care costs of FIC cats are much higher in comparison to rodents (Westropp and Buffington, 2002). Finally, because any cat with FIC in a study already has the syndrome, studies are limited to investigating the maintenance of the syndrome and cannot easily address the mechanisms underlying the initiation of the syndrome.

B.4 Cyclophosphamide Treatment

An alternative to direct instillation of noxious substances into the bladder to induce inflammation is intraperitoneal injection of cyclophosphamide (CYP). CYP is an antineoplastic agent with harsh side effects including hemorrhagic cystitis (Cox, 1979; Maggie et al., 1992). CYP is metabolized in the liver by P450 enzymes into mustard alkylating agents (Batsita et al., 2006). A urinary metabolite, acrolein, accumulates in the bladder and exerts a toxic effect by direct contact with the mucosal surface of the bladder. Acrolein induces histological and functional changes in the bladder similar to those seen in patients with IC/BPS. Histologic features include edema, vascular permeability, and

hemorrhage (Boucher et al., 2000; Bautista et al., 2006). Functionally, acrolein causes decreased bladder capacity, increased void frequency and somatic hypersensitivity (Guerios et al., 2006; Klinger et al., 2008; Studeny et al., 2008). Additionally, Eichel et al. (2001) showed that CYP increases urothelial permeability that lasted for 2 weeks after treatment. Because IC/BPS is a chronic and not an acute syndrome, such long term side effects resulting from CYP treatment correlate well with IC/BPS symptoms lending to its ability to model inflammation associated with IC/BPS.

The hyperreflexia and somatic sensitivity resulting from acrolein treatment are thought to be mediated, at least in part, by activation of capsaicin- sensitive c-fiber afferents (Maggie et al., 1992; Ahluwalia et al., 1994). This activation of c-fibers is similar to the “awakening of c-fibers” that mediates a variety of bladder dysfunction symptoms as described earlier. The effects of acrolein are mediated through transient receptor potential (TRP) channels, TRPA1 and/or TRPV1 (Dinis et al., 2004; Bautista et al., 2006; Wang et al., 2008). TRPA1 is activated by isothiocyanate or thiosulfinate compounds, the pungent components of mustard oil and garlic. TRPV1 is activated by heat and capsaicin, the pungent component of chili peppers. *In vitro*, acrolein application elicits depolarizing currents in TRPA1 positive neurons, but not TRPV1 positive neurons (Bautista et al., 2006). However, CYP-treated TRPV-1 knockout mice fail to develop hindpaw mechanical hypersensitivity that wild type counterparts exhibit (Wang et al., 2008). Additionally, an endogenous TRPV1 agonist, anandamide, increases in urinary bladder with CYP-induced cystitis and direct application to the serosal surface causes increased bladder reflex activity (Dinis et al., 2004). Activation of primary

sensory neurons can lead to central or peripheral release of neuropeptides, vasodilation and other neurogenic inflammatory effects, which in turn contribute to somatic hypersensitivity and bladder hyperreflexia (Maggie et al., 1992; Ahluwalia et al., 1994; Gepetti et al., 2008). In fact, bladder biopsies from patients with IC/BPS demonstrate elevated neuronal TRPV1 levels and that TRPV1 density correlates with pain scores (Mukerji et al., 2006).

Although the CYP model of bladder inflammation is well characterized and commonly used in current literature, it does not completely model symptoms of IC/BPS. The gross inflammation produced by CYP is more extreme than observed in the majority of bladders from IC/BPS patients. Additionally, the model does not enable researchers to address the underlying causes of urinary bladder inflammation. However, the model does allow scientists to identify chemical mediators of inflammation in the urinary bladder and to examine how these changes may affect bladder function. Additionally, the relative ease of the model makes it an attractive alternative in the laboratory. For example, CYP can be injected intraperitoneally and does not require the use of transurethral catheters and even with a non-localized injection site, visceral inflammation is limited to the bladder, while other viscera are spared (Boucher et al., 2000). Therefore, induction of bladder inflammation using CYP is relatively simple, inexpensive, precise and reproducible.

IC/BPS Symptoms	Model				NGF-OE
	Noxious Chemicals	FIC	Urothelial damage-PS	CYP	
Frequency	Yes	Yes	Yes	Yes	Yes
Referred Pain	?	Yes	?	Yes	Yes
↑ Urothelial Permeability	Yes	Yes	Yes	Yes	?
↑ NGF	?	Yes	?	Yes	Yes
↑ Inflammatory mediators	Yes	Yes	?	Yes	?
↑ suburothelial nerve plexus fibers	?	?	?	No	Yes

Table 1: Models of Urinary Bladder Inflammation as they relate to symptoms of IC/BPS

C. Inflammation and Plasticity within the LUT and Innervation

Plasticity within micturition reflex pathways leading to bladder hyperreflexia and associated pain includes changes in neurochemical, electrophysiological and organizational properties of primary bladder afferents and central connections. Numerous studies have demonstrated the ability of inflammation to induce such changes in visceral afferent pathways, including CYP-induced changes in bladder reflex circuitry (Figure 3).

Changes in the neurochemical properties of micturition reflex pathways may contribute to abnormal bladder function and associated pain. CYP-induced bladder inflammation produces plasticity in hormonal, peptidergic, neurotrophic and purinergic chemical profiles in sensory and autonomic motor innervation of the urinary bladder. Corticotropin releasing hormone (CRH) is a hormone and neurotransmitter involved in stress responses. CRH is present in the urinary bladder, SPN, and the suburothelial nerve plexus, and expression increases in these locations with CYP-induced cystitis (LaBerge et

al., 2006). CRH receptor -2 is also present in the suburothelial nerve plexus and urothelium of rat urinary bladders, and this expression increases following CYP-induced cystitis. These findings are interesting considering evidence for a link between stress and symptom onset and severity in IC/BPS patients (Rothrock et al., 2001). Developmental studies have demonstrated that changes in CRH expression may underlie the emergence of mature voiding reflexes (LaBerge et al., 2006; LaBerge et al., 2008). Functionally, exogenous application or genetic over expression of the CRH protein produces bladder hyperreflexia (Klausner et al., 2005; Million et al., 2007), and CRH antagonists can attenuate this effect (Klausner et al., 2005). Therefore CRH expression may mediate functional plasticity in micturition reflex circuitry under developmental and pathological conditions.

Expression of several neuropeptides changes in micturition reflex pathways with bladder inflammation. Following CYP-induced cystitis, bladder associated DRG and spinal cord cells with basal expression demonstrate increased amounts of neuropeptides such as CGRP, SP, pituitary adenylate cyclase-activating polypeptide (PACAP) and galanin in (Vizzard, 2000; Vizzard, 2001; Zvarova and Vizzard, 2006). Additionally, bladder associated DRG and spinal cord segments display a higher percentage of neuropeptide expressing cells after CYP-induced bladder inflammation (Vizzard, 2000; Vizzard, 2001). In the spinal cord, neuropeptide expression increases in the superficial DH (lamina I and II), LCP, DCM, SPN and IML (Vizzard, 2000; Vizzard, 2001; Zvarova and Vizzard, 2006).

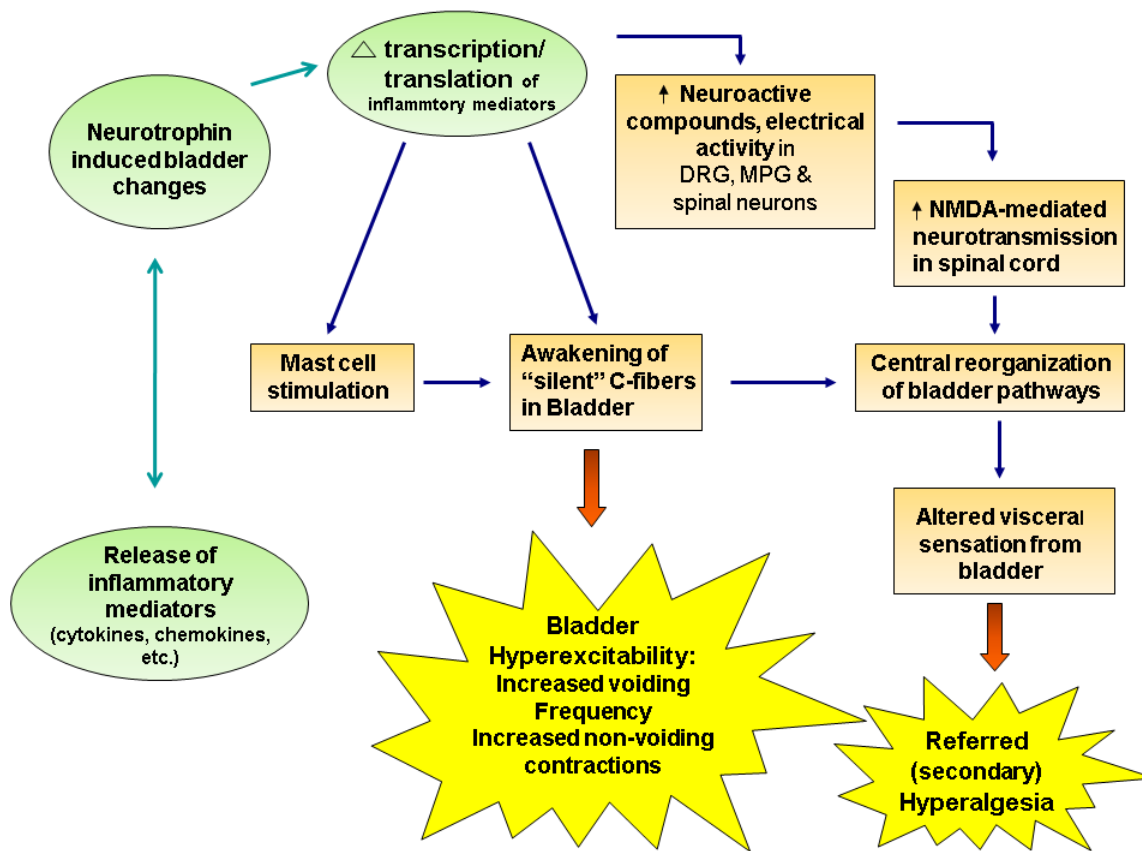


Figure 3. Physiologic events leading to symptoms of IC/BPS. Changes in neurotrophin or cytokine/ chemokine signaling can initiate a cascade of events leading to bladder hyperreflexia and referred somatic hyperalgesia.

Neurotrophic changes following CYP-induced bladder inflammation also occurs. Neurotrophins (e.g. NGF, brain-derived neurotrophic factor (BDNF)) and trophic receptors TrkA, TrkB and p75^{NTR} are present throughout micturition reflex pathways including the MPG, suburothelial nerve plexus and bladder associated DRG and spinal cord segments in control animals. Expression of neurotrophins and associated receptors increases following CYP-induced cystitis (Qiao and Vizzard, 2002; Murray et al., 2004; Klinger and Vizzard, 2008).

Functional purinergic receptor expression increases in bladder sensory afferents with bladder inflammation (Dang et al., 2008). Dissociated neurons from rats with CYP-induced cystitis demonstrate increased P2X receptor expression. Additionally, more fibers respond to purinergic agonists in comparison to neurons from animals without bladder inflammation (Dang et al., 2008). Alterations of purinergic signaling, together with other neurochemical changes, may contribute to the sensitization of bladder reflex pathways potentially provoking bladder hyperreflexia and pain.

Bladder inflammation also alters the electrical properties of bladder afferent neurons. After CYP-treatment, bladder afferent fibers exhibit a lower threshold for action potential generation and increased firing in response to isotonic distention (Yoshimura and de Groat, 1999; Dang et al., 2008; Yu and de Groat, 2008). CYP treatment increases receptive field size and causes a significant hypertrophy of afferent cell bodies in L6-S1 DRG (rat) (Dang et al., 2008). These data clearly demonstrate the ability of inflammation to change electrophysiological properties of neurons in the

sensory limb of micturition circuitry. Electrophysiological plasticity could translate to dysfunctional bladder symptoms such as hyperreflexia.

Organizational changes in micturition reflex pathways induced by CYP occur at the level of the urinary bladder and its central connections. CYP treatment increases CGRP- positive sensory afferent fibers in the bladder urothelium and detrusor smooth muscle and parasympathetic innervation in the urothelium (Dickson et al., 2006). Studies also demonstrate inflammatory-induced central changes such as an increase in post synaptic connections (Vizzard and Boyle, 1999; Vizzard, 2000). Growth- associated protein-43 (GAP-43) is a calmodulin-binding protein associated with synapse formation. CYP-induced cystitis increases GAP-43 expression in central micturition processing areas such as the DCM, DH, LCP, SPN and IML of the spinal cord (Vizzard and Boyle, 1999). Additionally, the protein gene product of the immediate early gene c-fos (Fos), that marks postsynaptic activation of spinal cord neurons receiving afferent information from the lower urinary tract, increases in number and spatial location in appropriate bladder spinal cord segments. These alterations mimic noxious irritation in non-inflamed bladders suggesting that inflammatory mediated central changes in Fos expression may be involved in pain mechanisms (Vizzard, 2000). Together these data implicate that inflammatory-induced synaptic plasticity of micturition reflex pathways (e.g. neurochemical, electrophysiological, organizational) may contribute to functional symptoms of bladder inflammation such as bladder hyperreflexia and associated pain.

IV. The Urothelium as a Sensory Organ

Recently, the functional contribution of the urothelium has advanced beyond the view of a passive barrier and is now suggested to have “neuron-like properties such as plasticity, sensory transduction and signaling capabilities, especially in the context of bladder inflammation (Birder et al., 2001; Birder et al., 2005). Urothelial cells express numerous receptors similar to those found in DRG such as purinergic, norepinephrine, acetylcholine, neuropeptide- and protease-activated receptors, acid sensing ion channels, neurotrophin receptors and transient receptor potential (TRP) channels (Birder, 2006; Apodaca et al., 2007; Birder & de Groat 2007). Functional receptor expression enables the urothelium to respond to diverse stimuli including stretch/ distention of the urinary bladder, soluble factors, neuroactive compounds and changes in pH (Birder, 2006; Apodaca et al., 2007). In addition to receptive sensory capabilities, the urothelium also releases a variety of signaling molecules (Birder, 2006; Apodaca et al., 2007). The anatomical proximity of underlying structures including bladder afferent neurons in the suburothelial plexus, interstitial cells in the suburothelial lamina propria and detrusor smooth muscle suggests the possibility of reciprocal connection with the urothelium (Figure 4). In support of this hypothesis, studies have demonstrated that urothelial-derived chemical mediators can influence the activity of underlying bladder afferent neurons and detrusor smooth muscle (Birder et al., 1998; Templeman et al., 2002; Birder and de Groat, 2007; Ikeda et al., 2007; Andersson et al., 2008; Smith et al., 2008).

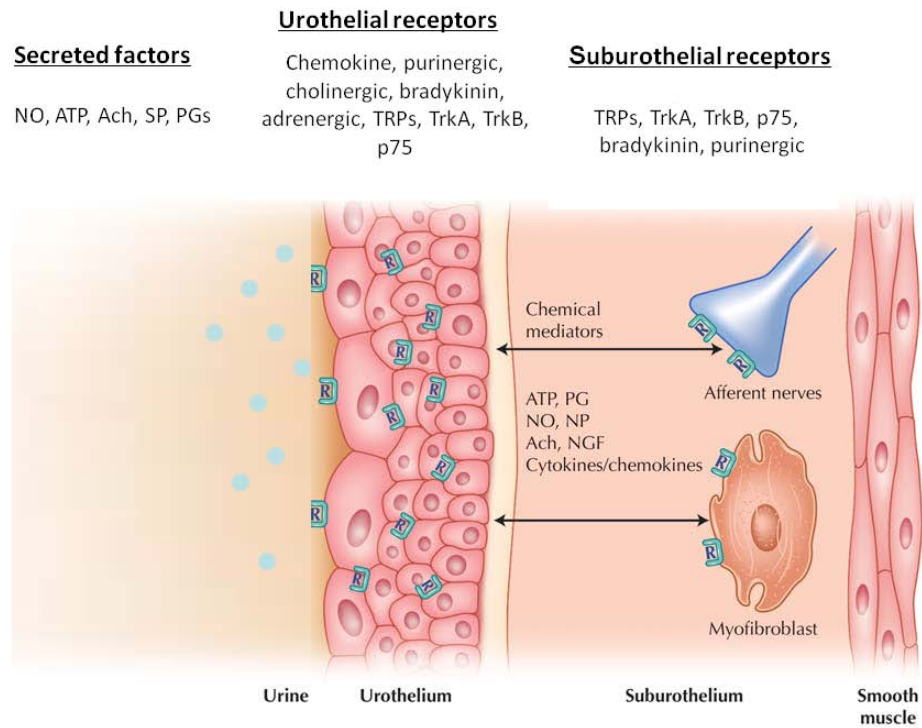


Figure 4. Schematic diagram illustrating the relationship of urothelial-derived factors and potential sites of receptor activation. Functional receptor expression and secretion capabilities enable the urothelium to respond to mechanical and chemical stimuli and to communicate with other tissues in the bladder. Substances released by the urothelium may activate receptors on urothelial cells, suburothelial plexus nerve fibers or myofibroblasts (interstitial cells). Modified from Arms et al., 2009.

For example, chemical or mechanical stimulation of the bladder facilitates the release of NO from urothelial cells which can then alter the activity of bladder afferent neurons (Birder et al., 1998; Andersson et al., 2008; Smith et al., 2008). Additional studies demonstrate that urothelial secreted factors modulate the spontaneous activity of detrusor smooth muscle (Templeman et al., 2002; Ikeda et al., 2007). These data suggest that the urothelium is a functional component of bladder sensory/ physiology.

Changes in chemical/receptor expression or secretion within the urothelium may mediate functional symptoms of various types of bladder dysfunction. For example, urine from IC/BPS patients contains elevated levels of ATP (Sun et al., 2001). ATP signals via ligand-gated ion purinergic receptors, specifically the P2X family. Urothelial cells and many sensory neurons terminating in the suburothelial plexus densely express a member of the P2X family, P2X₃ (Sun et al., 2001). In response to stretch, urothelial cells release ATP that can then bind P2X₃ receptors in bladder sensory neurons (Sun et al., 2001; Sun and Chai, 2004 and 2006). Bladders from IC/BPS patients show enhanced ATP release from urothelial cells in response to stretch (Sun et al., 2001), and increased expression of P2X₃ receptors in urothelial cells possibly enhancing purinergic signaling (Sun and Chai, 2004 and 2006). Additionally, P2X₃ agonists decrease the threshold for activation in bladder nociceptive neurons (Rong et al., 2002). Increased ATP release from urothelial cells combined with increased density of P2X₃ receptors may alter the bladder's response to distension effectively enhancing the afferent limb of the micturition reflex and leading to dysfunctional bladder symptoms such as hyperreflexia and associated pain.

The urothelium can be damaged by injury or inflammation (Birder and de Groat, 2007). When the urothelial barrier becomes compromised, proliferation and differentiation mechanisms attempt to restore the barrier integrity. Proliferation is thought to be responsible, at least in part, for the elevated levels of NGF in the bladder urothelium and urine from IC/BPS patients. As previously described, urinary bladder expression of NGF and associated receptors, TrkA, TrkB and p75^{NTR} is modulated with CYP-induced bladder inflammation (Vizzard, 2000; Murray et al., 2004; Klinger and Vizzard, 2008). Of substantial importance, transgenic mice over-expressing NGF achieved through the use of a urothelium-specific uroplakin II promoter exhibit phenotypic changes in the LUT similar to those shown in various forms of bladder dysfunction including IC/BPS and overactive bladder syndrome (Schnegelsberg et al., 2010). These changes include hyperinnervation of the suburothelial plexus, decreased bladder capacity and referred somatic sensitivity in the pelvic region. These studies demonstrate that urothelial-derived chemical mediators can contribute to functional changes in the urinary bladder and its innervation leading to hyperreflexia and associated pain. Additional studies demonstrate CYP-induced changes in urothelial expression and function of other neurochemicals such as neuropeptides (e.g., PACAP) and associated receptors (Girard et al., 2008), chemokines (Yuridullah et al., 2006; Vera et al., 2008) and signaling molecules (Corrow and Vizzard, 2007; Chung et al., 2010). Together, these data suggest that molecular changes within the organ itself (i.e. non-neuronal) can contribute to sensory and functional abnormalities including those extending beyond the scope of the bladder such as referred somatic pain to the body wall. This dissertation will

focus on inflammatory-induced molecular plasticity in the urinary bladder and how these changes mediate symptoms of bladder dysfunction.

V. The Role Chemokines in IC/BPS

Using the CYP-induced bladder inflammation model, we aim to characterize further the role of immune mediators in the development and/or maintenance of neuronal sensitization and chronic pain states associated with IC/BPS. Mechanical nerve injury, inflammatory insult and infection are all events that conjure immune activation. Chemical (e.g. cytokines, chemokines) and cellular (e.g. mast cells, leukocytes) mediators of the immune response are implicated in the initiation and maintenance of chronic pain states (White et al., 2007; Jung et al., 2008). The immune response consists of two stages: the innate response and the acquired response. Innate immunity initiates the body's immediate and general reaction to pathogen infiltration which includes, but is not limited to, the activation of phagocytic cells such as macrophages and neutrophils. Adaptive immunity on the other hand, because it relies on immunological memory of previous exposures to similar pathogens, responds much more slowly, is highly specific and offers future protection against similar pathogen infiltration. Evidence suggests that mediators of the innate immune response play a role in the development of abnormal pain states (White et al., 2007; Jung et al., 2008). Receptors expressed by immune cells recognize conserved molecular patterns of infectious agents (e.g., bacteria, viruses) or cellular debris (e.g., as generated during processes such as wallerian degeneration) and respond by increasing cytokine synthesis and secretion (White et al., 2007). Cytokines spark a cascade of immunological events that involves production of more inflammatory

mediators in other cell types and extravasation of leukocytes to the site of injury/ inflammation. The immune response acts to heal the injured/ inflamed area and also to sensitize nociceptive neurons, thus increasing the pain response in order to prevent further insult (White et al., 2007). While initial immune activation and sensitization of sensory neurons is protective, prolonged immune activity and sensory sensitization occurring after tissue healing are associated with chronic pain syndromes and are suggested to underlie mechanisms of neuropathic pain. Neuropathic pain persists in the absence of physiologic neuronal stimuli and often manifests in the form of hyperalgesia or allodynia. Chronic neuropathies are common in patients who suffer from diseases or injury such as human immunodeficiency virus (HIV) (Oh et al., 2001), MS (Haensch and Jörg, 2006), SCI (Yoshimura, 1999) and IC/BPS (Erikson, 1999). Unfortunately, pain management becomes problematic because neuropathic pain is often resistant to non-steroidal anti-inflammatory drugs (NSAIDs) and opioid treatment (e.g. morphine).

A. Overview of Chemokines

Potential chemical mediators responsible for nociceptive sensitization include chemokines, otherwise known as chemotactic cytokines. Chemokines are a large family of structurally and functionally related proteins that are important mediators of immune responses, inflammatory processes and nociception. In the immune response, chemokines facilitate tissue recovery by causing the extravasation of leukocytes from blood plasma to the site of injury. Chemokine receptors present on leukocytes sense increasing chemotactic concentration gradients and facilitate cellular motility towards them (Rutkowski and DeLeo, 2002).

Approximately 100 amino acids in length, chemokines are small, secreted proteins that comprise 4 subfamilies: CC, CXC, CX3C and C (for review see White, 2005). Each subfamily is named for the first cysteine residue motif from its amino terminus. Families of chemokines exert their actions by binding to related G-coupled protein receptors (GPCRs). Within each subfamily, receptor and ligand pairing is not mutually exclusive; in multiple ligands can bind the same receptor and visa versa (for review see White, 2005). The complexity of chemokine receptor binding presents challenges when elucidating the functional role of chemokine signaling. Despite difficulties, unveiling the role of chemokines and their receptors in both physiological and pathological states could provide insights to possible therapeutic interventions in a variety of chronic pain conditions including IC/BPS.

B. Chemokines and Altered Sensory Processing

Inflammatory mediators such as proinflammatory cytokines (e.g., tumor necrosis (TNF)- α , interleukin (IL)-6, IL-1 β), COX-2, NGF, protons, prostaglandins and bradykinin have been implicated in the direct sensitization of nociceptive afferents (Cheng and Ji, 2008). Traditionally, chemokines were not thought to assert direct effects on primary sensory neurons. Rather, chemokine/ receptor interaction on the plasma membrane of leukocytes was thought to stimulate leukocyte-release of nociceptive mediators via GCPR signaling mechanisms (White et al., 2007). However, electrophysiological, expressional and functional pain studies have demonstrated the possibility of direct chemokine-mediated neuronal hypersensitivity and pain (Figure 5). For example, *in vitro*, exogenous chemokine application can physiologically alter sensory neurons by changing membrane

potentials (Sun et al., 2006), decreasing thresholds for action potential generation (Sun et al., 2006), increasing excitability and evoking discharges (White et al., 2005; Sun et al., 2006). Various chemokines including CXCL12 can modulate calcium ion currents in cultured DRG cells, potentially facilitating hyperexcitability (Oh et al., 2001; Oh et al., 2002; Bhangoo et al., 2007a and b). In a neuronal injury model, chronic compression of the DRG elicits a depolarizing response to chemokines that was not detected in control (non-compressed) DRG (White et al., 2005). These data suggest that direct chemokine action may contribute to altered neuronal function associated with neuropathies.

Under basal conditions, with the exception of CXCL12 (White et al., 2007), neuronal chemokine expression is sparse; however, following nerve injury or inflammation, expression of chemokines and associated receptors increases significantly in macrophages and infiltrating T-cells, but more importantly in sensory neurons and glia (Rutkowski and DeLeo, 2002; Tanaka et al., 2004; Verge et al., 2004; White et al., 2005; Bhangoo et al., 2007a, b and 2009, Jung et al., 2008). Additionally, increased neuronal activity, as would occur during injury/ inflammation, has been shown to induce chemokine transcription (Jung and Miller, 2008). Chemokine expression in, and subsequent secretion from the various cell types would enable chemokines, by diffusion, to interact with functional chemokine receptors on DRG neurons thus facilitating hyperexcitability changes such as those described above.

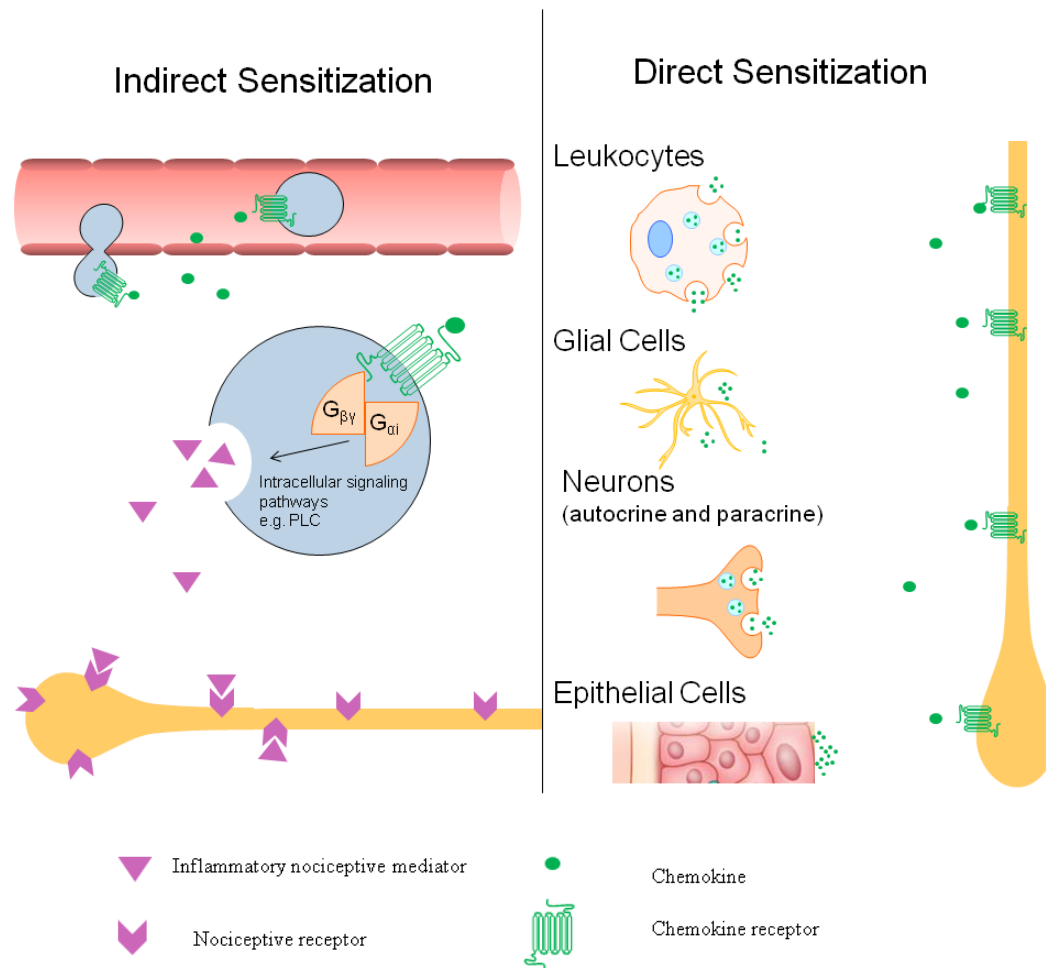


Figure 5. Indirect vs. direct neuronal modulation by chemokine signaling. After the initiation of extravasation, chemokines were thought to influence neuronal functioning indirectly by facilitating the release of other excitatory chemicals from leukocytes via GPCR downstream signaling pathways (left panel). However more recent evidence suggests that neurons released from leukocytes, glial cells, neurons or epithelial cells can directly sensitize neurons (right panel). Modified from Rittner et al., 2008.

In conjunction with expressional increases during pathological conditions, studies illustrate a strong relationship between chemokines and pain. Oh and colleagues (2001) published an early example of nociceptive chemokine function when they showed that intraplantar administration of various chemokines such as CXCL12, CCL5 and CCL22 induce mechanical hypersensitivity lasting for at least 3 hours. Since then numerous studies have reported that either exogenous peripheral (e.g. intradermal) or central (e.g. intrathecal) chemokine application induces mechanical hypersensitivity and/ or thermal hyperalgesia (Milligan et al, 2004; Tanaka et al., 2004; Qin et al., 2005; Zhang et al., 2005; Dansereau et al., 2008, Bogan et al., 2009). Intrathecal administration of CCL2 can produce mechanical hypersensitivity within 30 minutes and hyperalgesic effects can be detected up to 4 days after administration (Dansereau et al., 2008). In contrast, CCL2-induced thermal hypersensitivity resolves 24 hours after administration (Dansereau et al., 2008). Interestingly, transgenic mice lacking the CCR2 receptor (principle receptor for CCL2) are resistant to the development of mechanical hypersensitivity following mechanical nerve injury; however, following Complete Freund's Adjuvant (CFA)-induced neural inflammation, these mice display only a small, insignificant decrease in mechanical sensitivity compared to control animals and show no changes in thermal nociception (Abbadie et al., 2003). These data suggest specificity for chemokine function with respect to type of injury and pain modality.

Studies utilizing antagonists against chemokine signaling provide evidence for a therapeutic role with respect to neuropathic pain. In two different models of HIV-1 associated neuropathy, Bhangoo et al., (2007b and 2009) demonstrate that anti-retroviral

drug- or viral coat protein, gp120-, induced mechanical hypersensitivity is attenuated by acute, systemic treatment with CCR2 or CXCR4 antagonists (Bhangoo et al., 2007b). Other pain eliciting models such as focal demyelination, CFA- induced inflammation and partial sciatic nerve ligation have demonstrated the therapeutic effects of chemokine receptor antagonists (Milligan et al., 2004; Bhangoo et al., 2007a; Manjavachi et al., 2009).

One potential mechanism underlying chemokine nociceptive properties is sensitization of the TRPV1 receptor. *In vitro*, pre treatment of DRG neurons with chemokines enhances the response to capsaicin or anandamide (Jung et al., 2008; Zhang et al., 2005). Possible signaling mechanisms involved in chemokine-induced TRPV1 sensitization include PLC and PKC mediated signaling pathways (Zhang et al., 2005; Jung et al., 2008).

Various chemokines and receptors colocalize with neuronal TRPV1 as well as neuropeptides known be released in a TRPV1-dependent manner (Oh et al., 2001; Zhang et al., 2005; Dansereau et al., 2008; Jung et al., 2008). Notably, Jung et al. (2008) showed large dense-core vesicles containing both CCL2 and CGRP. Dansereau et al., (2008) demonstrate calcium-evoked release of CCL2 following incubation of DRG neurons with potassium or capsaicin. Functioning as a neurotransmitter-like molecule, vesicular chemokine release could assert pro-inflammatory and neuronal hyperexcitability effects in neighboring DRG cells or spinal cord dorsal horn neurons and glia (discussed later). Chemokines released from central primary afferent terminals could exert either direct activation of SDH neurons via functional chemokine receptor

expression or indirect sensitization via activation of microglia and astrocytes that subsequently release nociceptive mediators.

Heightened DRG neuronal activity may produce neurologic pain by increasing central input to the spinal cord. Repetitive firing of the DRG neuron could increase nociceptive neurotransmitter (e.g. substance P and CGRP) release into the dorsal horn of the spinal cord, therefore increasing activity of spinal neurons that mediate both local reflexes and ascend to higher brain centers. *In vitro*, chemokine application can evoke release of both SP and CGRP from neurons (Oh et al., 2001; Qin et al., 2005). Such events could potentially lead to a phenomenon known as “central sensitization” where intensely heightened peripheral input decreases thresholds necessary to elicit action potentials in DH neurons thus facilitating increased pain signal transduction to the brain (Oh et al., 2001; White et al., 2007). Increased primary afferent signaling may also induce organizational and neurochemical changes in central synapses underlying central sensitization and chronic pain syndromes.

B. Chemokines, Glial Activation and Pain

Altered sensory functioning is not limited to peripheral sensory neurons, but may also affect higher order neurons in the spinal cord and/or brainstem and brain (White et al., 2007). The central nervous system, once thought of as an immunologic devoid network, is now considered to play major role in inflammatory pain (Rutkowski and DeLeo, 2002). Activated astrocytes and microglia have the capability to respond to noxious stimuli by releasing inflammatory mediators such as cytokines and chemokines that can then diffuse to other cells including neurons and thus accentuate neuronal

activity (Rutkowski and DeLeo, 2002; Watkins and Maier, 2003; Tsuda et al., 2005; Milligan et al., 2008; Gao and Ji, 2010; Figure 6).

Astrocytes serve a regulatory role under physiologic conditions. Their juxtaposition to neuronal somas, dendrites and pre- and post- synaptic terminals enables them to monitor and modulate extracellular ion and neurotransmitter concentrations, availability of chemical precursors and pH (Watkins and Maier, 2003). Microglia are normally quiescent and express relatively few cell surface receptors until they become activated by invading pathogens or noxious stimuli (Watkins and Maier, 2003). The term activation represents a multitude of orchestrated expressional and functional changes that occur within a cell enabling it to engage in activities not previously possible (Watkins and Maier, 2003). Upon activation astrocytes and microglia undergo morphologic and functional changes enabling them to initiate pro-inflammatory cytokine production, signaling cascades and secretion of cytokines and neurochemicals (Watkins and Maier, 2003; Tsuda et al., 2005; Milligan et al., 2008; Gao and Ji, 2010; Figure 6). Activated astrocytes exhibit hypertrophy and an increase in the amount of intermediate filaments, specifically glial fibrillary acidic protein (GFAP), a marker of activated astrocytes (Garrison et al., 1991). Functionally, astrocytes increase cytokine production (Watkins and Maier, 2003). Microglia demonstrate retracted processes, hypertrophy and proliferation in response to activation. Microglia become phagocytic, migrate to areas of infection/ damage and upregulate cytokines and numerous cell surface receptors including chemokine, cytokine and purinergic (Watkins and Maier, 2003; Lindia et al., 2005).

A variety of functional receptors are present on astrocytes including NMDA and non NMDA ionotropic receptors, metabotropic glutamate receptors and purinergic and SP receptors (Milligan et al., 2008). Functional receptor expression enables astrocytes and microglia to respond to proinflammatory cytokines (i.e., TNF- α , IL-1 β , IL-6), SP, excitatory amino acids (EAAs; e.g., glutamate) and ATP (Figure 6). In response, glial cells release the same cytokines (Milligan et al., 2008) in addition to chemokines, (Rostene et al., 2007) neurotransmitters, nitric oxide (Burgher et al., 1997) and prostaglandins (Halassa et al., 2007; Pocock and Kettenment 2007; Figure 6).

Neurochemicals released by glial cells can diffuse away to directly excite sensory neurons and can lead to increased SP and EAA release from primary afferent neurons either centrally or peripherally (Malcangio et al., 1996; Aimar et al., 1998; Southall et al., 1998; Inoue et al., 1999; Guo and Schluesener, 2007). Alternatively, glial-derived neurochemicals can indirectly excite neurons by inducing transcription of pro-nociceptive mediators such as COX-2 (increases production of prostaglandins) in neurons or other glial cells resulting in further enhancement of the inflammatory response and pain signal transduction (Milligan et al., 2008).

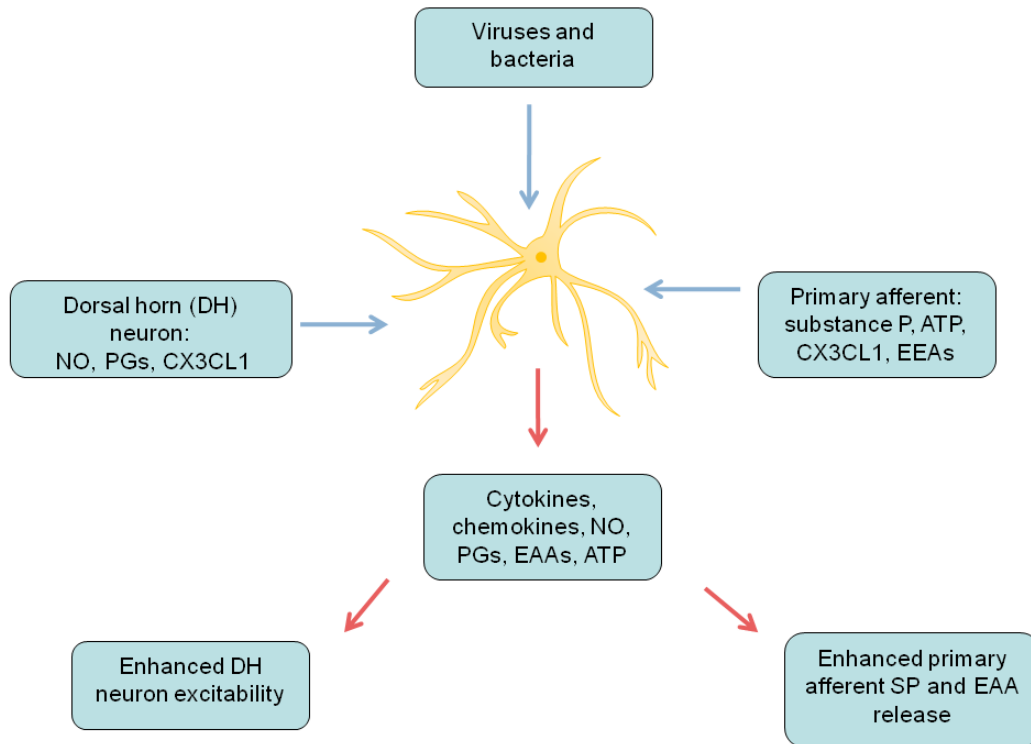


Figure 6. Schematic diagram illustrating the relationship of activated glial cells and inflammatory signaling molecules. Mediators causing glial activation include viruses and bacteria or substances released by primary afferent or spinal tract neurons. NO, nitric oxide; DH, dorsal horn; PGs, prostaglandins; EAAs, excitatory amino acids; SP, substance P; ATP, adenosine triphosphate. Modified from Watkins and Maier, 2003).

Garrison and colleagues (1991) were the first to correlate glial activation and nociception. They showed that increased glial activation coincides with hyperalgesia following peripheral nerve injury. Since then numerous studies have demonstrated that neuropathic pain resulting from inflammation or injury to peripheral tissues can be blocked with pharmacological agents that disrupt glial viability (Meller et al., 1994; Watkins et al., 1997; Milligan et al., 2000; Raghavendra et al., 2003; Watkins and Maier, 2003).

Chemokines have been investigated for their potential role in glial activation because they are putative signaling molecules of the immune system and contribute to pain process, therefore their presence in astrocytes or microglia should not be surprising. Indeed chemokines and receptors are detected in dorsal horn neurons and activated astrocytes and microglia in numerous models of neuropathic pain including peripheral or central nerve damage or tissue inflammation (Harrison et al., 1998; Bajetto et al., 1999; Mennicken et al., 1999; Bajetto et al., 2002; Cartier et al., 2005; Mines et al., 2007; Gao and Ji, 2010). Blockade of chemokine signaling reduces hyper-nociceptive pain behaviors after injury/inflammation (Milligan et al., 2004; Bhangoo et al., 2007; Manjavachi et al., 2009).

Chemokines may act as relay messengers, communicating back and forth between glial cells and neurons inducing pro-inflammatory effects such as neuronal hyperexcitability and neuronal and/ or glial release of neurochemicals. Chemokine expression in glial cells is thought to be driven by pro-inflammatory cytokines, TNF- α , IL-1 and IL-6 (Milligan et al., 2004; Milligan et al., 2008; Gao et al., 2009). Exposure of

astrocytes to TNF- α , a pro-inflammatory cytokine, induces a substantial (>100-fold) increase in CCL2 expression and secretion (Gao et al., 2009; Gao and Ji, 2010). Considering that CCR2 receptors are constitutively expressed on dorsal horn neurons and the juxtaposition of astrocytes to neurons, glial-released CCL2 could, by simple diffusion, bind to receptors on dorsal horn neurons (Gosselin et al., 2005; Gao et al., 2009). Chemokine signaling on sensory neurons may induce electrophysiological (Gao et al., 2009), transcriptional (Ye, 2001) neurochemical (Gao et al., 2009) and organizational changes that underlie chronic pain phenotypes.

Communication between glial cells and neurons is suggested to be reciprocal (Milligan et al., 2008; Gao and Ji, 2010). As such, neuronally-derived chemokines can signal to glial cells in the opposite direction (Milligan et al., 2008; Gao and Ji, 2010). CX3CL1 (a.k.a. fractalkine) is a striking example of this phenomenon. In the CNS, fractalkine is expressed on the cell surface of neurons only; the fractalkine receptor however, is expressed only on microglial cells in the CNS (Harrison et al., 1998; Watkins and Maier, 2003; Verge et al., 2004; Lindia et al., 2005; Milligan et al., 2008). Fractalkine exists in both membrane-bound and soluble forms. Under physiologic conditions, fractalkine most often binds its cognate receptor CX3CR1 in the membrane-bound form. However, increased neuronal firing increases fractalkine shedding via matrix metalloproteinase mechanisms and the soluble form of fractalkine can diffuse away and bind receptors on microglial cells (Chapman et al., 2000; Watkins and Maier, 2003; Milligan et al., 2008). Fractalkine signaling has been associated with inflammatory neuropathological diseases such as MS (Krumbholz et al., 2006) and amyotrophic lateral

sclerosis (ALS) (Milligan et al., 2008). Chemokine signaling between bladder sensory afferents and glial cells could modulate symptoms of IC/BPS especially considering that peripheral injury or inflammation (e.g., bladder) can induce central glial activation.

D. Chemokines and Visceral Inflammation

Both rodent and clinical studies assessing patients with various pelvic inflammatory/ pain syndromes indicate a role for chemokines in the initiation or maintenance of visceral inflammation. For example, in a model of chemically-induced colitis, blockade of CXCR2 signaling reduces neutrophil influx, myeloperoxidase (MPO) activity, iNOS and COX-2 expression and infiltration of cytokines and chemokines (Bento et al., 2008). CXCR4 peripheral T-cell expression is increased in patients with ulcerative colitis and expression levels correlate with disease activity (Mikami et al., 2008). Additionally, chemically-induced colitis in mice leads to an increase of CXCR4-positive leukocytes and CXCL12 expression in colonic tissue (Mikami et al., 2008). Administration of a CXCR4 antagonist reduces these inflammatory effects (Mikami et al., 2008). Elevated chemokine levels have been detected in the seminal plasma of patients with chronic prostatitis or chronic pelvic pain syndrome (Penna et al., 2007). Similarly, patients with IC/BPS exhibit elevated chemokine expression in bladder lymphocytes and mice treated with CYP display similar expressional patterns (Sakthivel et al., 2008). Blockade of CXCL10 signaling reduces severity of CYP-induced bladder inflammation by reducing hyperplasia, pithelial erosions and infiltration of T cells, mast cells and killer T cells in the bladder urothelium of rats (Sakthivel et al., 2008).

Urothelial chemokine signaling may contribute to inflammatory related bladder hyperreflexia. Agace et al., (1993) demonstrate an early example of urothelial-derived chemokine expression; following exposure to *Escherichia coli*, urothelial cells produce copious amounts of CXCL8. Similarly, urothelial expression of chemokines such as CX3CL1 and CXCL12 and associated receptors, CX3CR1 and CXCR4 respectively, increases in the urothelium after CYP-induced cystitis in rodents (Yuridullah et al., 2006; Vera et al., 2008). Considering the extensive data implicating a sensory and signaling role for the urothelium, it is possible that urothelial-derived chemokines contribute to symptoms of bladder dysfunction.

E. Chemokines and Opioid Desensitization

Chronic neuropathic pain management associated with IC/BPS, SCI or HIV-associated neuropathy is problematic because of its tendency to develop tolerance to opioid drugs (e.g. morphine). Opioid receptors (subtypes μ , δ and κ) are GCPRs that are present on the plasma membrane in sensory neurons (Zhang et al., 2004). Opioid signaling initiates a variety of downstream signaling events via G-protein signaling mechanisms including activation of inward rectifying potassium channels and inhibition of calcium channels (Zhang and Oppenheim, 2005). These effects block neuronal membrane depolarization, action potential generation, neurotransmitter release and therefore pain signals fail to propagate and analgesia is observed (Zhang and Oppenheim, 2005). A phenomenon termed “heterologous desensitization” describes the ability of one type of receptor to inhibit the actions of a different receptor, usually via inhibition of G-protein activity (Zhang and Oppenheim, 2005), and may explain the tolerance to

opioid substances that occurs in the presence of neuropathic pain. Chemokine receptors are also coupled to G-proteins and expressed on the cell surface of neurons. *In vitro*, chemokine application prior to an μ -opioid agonist, DAMGO, induces μ -opioid receptor internalization as evidenced by a reduction in the amount of cell surface expression (Zhang et al., 2004). *In vivo*, DAMGO asserts analgesic effects in the cold water tail-flick test by increasing the time until a rat removes its tail from the cold water stimulus (Szabo et al., 2002). Application of a variety of chemokines into the PAG prior to DAMGO treatment reverses the DAMGO-induced tail-flick latency (Szabo et al., 2002). Chemokine signaling may contribute to neuropathic pain not only by sensitizing afferent neurons but also activating glia or desensitizing the body's anti-pain mechanisms.

VI. Involvement of PI-3K/ AKT Signaling in Inflammatory Pain

A. PI-3K/AKT Signaling Overview

Phosphoinositide 3-kinase (PI-3K) signaling via AKT is a common cellular signaling pathway that exerts numerous functions and is present in a variety of cell types (for review see Scheid and Woodgett, 2001). PI-3K functionally activates AKT by indirect phosphorylation (Burgering and Coffey, 1995). Extracellular stimuli activate cell surface receptors that are coupled to intracellular signaling pathways such as PI3-K. Activated PI3-K then phosphorylates membrane inositol phospholipids to generate $PI(3,4)P_2$ and $PI(3,4,5)P_3$. $PI(3,4)P_2$ and $PI(3,4,5)P_3$ act as landing sites for both AKT and phosphoinositide-dependent kinase (PDK1). Both AKT and PDK1 have pleckstrin homology (PH) domains that target them to $PI(3,4)P_2$ and $PI(3,4,5)P_3$ in the plasma membrane. After binding the membrane inositol phospholipids, AKT and PDK1 undergo

conformational changes, which then, due to close proximity, allow PDK1 to phosphorylate (activate) AKT at both serine (473) and threonine (308) sites. Finally, AKT dissociates from the plasma membrane into the cytosol where it will play a role(s) in other signaling cascades (Scheid and Woodgett, 2001).

B. PI-3K/AKT Signaling in Altered Sensory Processing

Although AKT is best known for its role in cell survival via anti-apoptotic effects, recent studies have demonstrated novel roles for AKT signaling in altered sensory/pain processing. For example, basal phosphorylated (p) AKT expression is present in rodent lumbosacral DRG (Sun et al., 2007; Shi et al., 2009) and superficial laminae of the spinal cord (Sun et al., 2006; Guedes et al., 2008) and significantly increases following peripheral nerve/ tissue injury or inflammation (Sun et al., 2006; Sun et al., 2007; Xu et al., 2007; Guedes et al., 2008; Pezet et al., 2008; Shi et al., 2009). Additionally, colocalization of pAKT with markers of unmyelinated pain transducing c-fibers such as isolectin- IB4, TrkA, CGRP and TRPV1 (Zhuang et al., 2004; Sun et al., 2007; Xu et al., 2007; Shi et al., 2009) has been demonstrated. Specific inhibitors of AKT signaling can attenuate mechanical hyperalgesia resulting from peripheral nerve injury or inflammation (Sun et al., 2006; Sun et al., 2007; Xu et al., 2007) suggesting a nociceptive role for AKT signaling. Both prophylactic treatment (i.e. administered prior to peripheral nerve injury/ inflammation) and therapeutic treatment (administered after peripheral nerve injury/ inflammation and the development of hyperalgesia) with inhibitors of AKT phosphorylation reduce peripheral injury-/ inflammation- induced pain. Therefore AKT

is thought to play a role in both the initiation and maintenance of peripheral hyperalgesia (Sun et al., 2006; Xu et al., 2007).

In addition to the mediation of somatic pain processes, AKT signaling may also mediate visceral inflammation. After chemically-induced colitis in rodents, basal pAKT expression in the superficial dorsal horn increases significantly (Qiao and Grider, 2009). Furthermore, urothelial pAKT expression has been detected in mice, thus raising the possibility of pAKT participation in urothelial-mediated effects on bladder sensory physiology (Tamarkin et al., 2006). As a result, PI-3K/AKT signaling may be a target for therapeutic intervention in various peripheral injury/ inflammatory conditions, including IC/BPS.

Potential upstream effectors of AKT activation include NGF and other growth factors such as platelet derived growth factor, epidermal growth factor and insulin-like growth factor, interleukins, cytokines and reactive oxygen species (Datta et al., 1999). For the purpose of this dissertation, only NGF and chemokine regulated AKT phosphorylation will be discussed because of their relevance to bladder dysfunction.

NGF interactions with the high affinity receptor, TrkA, are known to activate PI3-K/ AKT pathways (Datta et al., 1999). Elevated NGF levels are detected in the urothelium and urine from patients with IC/BPS (Lowe et al., 1997; Okragly et al., 1999). Numerous rodent studies demonstrate the functional capacity of elevated NGF signaling in the bladder because they demonstrate that exogenously- or endogenously- induced elevated NGF results in sensitization of bladder afferents and bladder hyperreflexia (Dmitrieva et al., 1997; Chuang et al., 2001; Lamb et al., 2004; Yoshimura et al., 2006;

Zvara and Vizzard, 2007; Schnegelsberg et al., 2010). CYP-induced bladder inflammation modulates the expression of NGF and TrkA in the urinary bladder (Vizzard, 2000; Murray et al., 2004), bladder primary afferents (Qiao and Vizzard, 2002) and major pelvic ganglia (Murray et al., 2004); therefore, activation of TrkA may contribute to phosphorylated AKT expression in the urinary bladder or micturition reflex pathways during bladder inflammation. Other NGF/TrkA downstream effectors including extracellular signal-regulated kinase (ERK)1/2 (Corrow and Vizzard, 2007; Chung et al., 2010) and c-Jun N-terminal kinase (JNK) (Chung et al., 2010) increase in the urinary bladder in an NGF-dependent manner following CYP-induced cystitis (Chung et al., 2010). Corrow and Vizzard (2007) demonstrated that inhibition of ERK phosphorylation significantly reduces CYP-induced bladder hyperreflexia in rats, therefore, NGF-mediated signaling may potentiate phenotypic symptoms associated with bladder dysfunction.

C. Chemokine Influence on PI-3K/AKT Signaling

Inflammation induced PI-3K signaling may be a direct effect of inflammatory mediators themselves. For example, chemokines regulate AKT phosphorylation in normal processes such as brain development (Bajetto et al., 2001) and lymphopoiesis (Tilton et al., 1997 and 2000), as well as in disease states such as HIV (Poggi et al., 2004), diabetes (Liu et al., 2008), breast cancer (Fernandis et al., 2004), and brain tumorigenesis (Bajetto et al., 2001). In particular, CXCL12 promotes AKT phosphorylation in a variety of cell types including lymphocytes (Tilton et al., 2000), astrocytes (Bajetto et al., 2001), hepatocytes (Liu et al., 2008) and breast, endometrial

and prostate cancer cell lines (Fernandis et al., 2004; Wang et al., 2005; Zhao et al., 2006). Therefore it is possible that chemokines can influence PI-3K/AKT signaling in bladder cells. Noxious stimulation of the bladder either by mechanical stretch or infection induces PI-3K/AKT signaling in smooth muscle cells (Adam et al., 2003; Stover and Nagatomi, 2007) or the urothelium (Tamarkin et al., 2006), but the role of chemokine regulation is not known.

As described earlier, various chemokines, including CXCL12, can modulate calcium ion currents and increase excitability in a number of cells including primary DRG neurons (Oh et al., 2001; Oh et al., 2002; Nelson and Gruol, 2004; White, 2005; Bhangoo et al., 2007). Additionally, the PI-3K/AKT signaling pathway can be activated via neuronal activity or calcium ion influx in a variety of cell types such as bladder smooth muscle cells (Stover and Nagatomi, 2007), striatal neurons (Perkinton et al., 2002), sympathetic neurons (Vaillant et al., 1999) and DRG neurons (Pezet et al., 2005). Chemokine induced neuronal hyperexcitability could potentially lead to activation of the PI3-K/ AKT signaling pathway. Considering that chemokines, chemokine receptors and AKT have been detected in sensory afferents, an inflammatory-induced activation of AKT seems plausible (Oh et al., 2001; Tanaka et al., 2004; Verge et al., 2004; Pezet et al., 2005; Zhang et al., 2005).

D. AKT and TRPV1 Sensitization

One mechanism phosphorylated AKT may contribute to nociceptive processes is via sensitization of the TRPV1 receptor. Either neurotrophin- or chemokine- induced activation of AKT could lead to TRPV1 upregulation or sensitization. For example, PI3-

K activation has been implicated in NGF-induced TRPV1 expression and trafficking to the plasma membrane (Chuang et al., 2001; Bron et al., 2003; Zhang et al., 2005; Stein et al., 2006). AKT is not the sole downstream target of PI3-K activation therefore its role in NGF-mediated TRPV1 trafficking or expression, while plausible, is not definitive. Similarly, studies demonstrate that direct NGF-induced TRPV1 sensitization (i.e. increased intracellular cation flux) can be mediated by each of the major NGF/ TrkA downstream signaling pathways, PI3-K, MAPK and PLC; however, there is no consensus on the contribution of each pathway alone, or in combination, on NGF-evoked TRPV1 sensitization.

E. AKT and Central Sensitization

Central sensitization involves a number of changes within with spinal cord circuitry, resulting from high frequency input from peripheral primary neurons that renders central nociceptive neurons hyperexcitable (Man et al., 2003; Pezet et al., 2008). The molecular changes underlying central sensitization are similar to those observed in other forms of synaptic plasticity such as long term potentiation (LTP) in the hippocampus, the major mechanism thought to underlie learning and memory. Similarities include increased intracellular calcium flux through N-methyl-D-aspartic acid (NMDA) receptors (NMDARs) that leads to functional changes of post-synaptic α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors (AMPA) receptors (AMPA) (Bliss and Collingridge, 1993; Zhuo and Hawkins, 1995; Malenka and Nicoll, 1999; Soderling and Derkach, 2000; Ji et al., 2003; Man et al., 2003; Pezet et al., 2008). Furthermore, PI3-K signaling has been implicated in the development of neuronal properties associated

with LTP. For example, PI3-K signaling is necessary for high frequency stimulus (HFS)-induced LTP in the hippocampus and amygdala (Lin et al., 2001; Sanna et al., 2002; Opazo et al., 2003; Karpova et al., 2006). Inhibitors of PI3-K block NMDAR downstream signaling effects such as activation of MAPK signaling pathways (Pezet et al., 2008) and GluR1 AMPAR subunit trafficking to the plasma membrane (Man et al., 2003; Pezet et al., 2008). Increased activation of AKT occurs concomitantly with PI3-K-dependent LTP and therefore may contribute to the development or maintenance of LTP (Lin et al., 2001; Sanna et al., 2002; Karpova et al., 2006). For example, blockade of AKT phosphorylation reduces the number of postsynaptic excitatory potentials (EPSPs) after HFS-induced LTP (Karpova et al., 2006). Due to similarities between underlying mechanisms of LTP in the brain and central sensitization in the spinal cord, AKT may contribute to plasticity within DH synapses leading to central sensitization and ultimately to heightened pain signal transduction.

VII. Summary

Cellular sources of chemokines and phosphorylated AKT include urothelial (Tamarkin et al., 2006; Yuridullah et al., 2006), inflammatory (Mikami et al., 2008; Sakthivel et al., 2008), glial (Lindia et al., 2005) and neuronal cells (Verge et al., 2004; Zhuang et al., 2004). After injury or inflammation, expressional increases or secretion from any of these cell types may lead to neuronal hypersensitivity facilitating pain signal transduction (Oh et al., 2001; Karpova et al., 2006). Neuronal sensitization may result from direct enhancement of cation channel activity (e.g. TRPV1) (Zhu and Oxford, 2007) or indirectly via transcriptional mechanisms (Watkins and Maier, 2003; Bento et al.,

2008). Increased peripheral sensory activity enhances central and peripheral SP and CGRP release which will ignite further inflammatory cascades in glial cells (centrally) or leukocytes (peripherally) (Oh et al., 2001; Qin et al., 2005). Such signaling mechanisms would lead to a cyclic inflammatory/ neural sensitization response with no clear “stop” signal. These inflammatory-induced changes may contribute to chronic hyperreflexic bladder and pain symptoms associated with bladder inflammatory conditions such as IC/BPS.

VIII. Project Aims and Hypotheses

The purpose of this dissertation study was to evaluate the expression of chemical mediators and associated signaling pathways in urinary bladder inflammation and how these mediators relate to micturition function/ dysfunction. The overall hypothesis for this proposal is that changes at the level of the urinary bladder can result in altered bladder sensory physiology that results in a hyperactive bladder state. Specifically, we examined the expression of the chemokine/receptor pair, CXCL12/CXCR4, in the urinary bladder both in the presence and absence of CYP-induced inflammation. We also evaluated the contribution of CXCL12/CXCR4 signaling to functional micturition in control rats and those treated with CYP. The activation (phosphorylation) of a ubiquitous signaling molecule, AKT was also examined in control and inflamed rat urinary bladders and its functional contribution to micturition was evaluated in control and CYP-treated rats.

Clinical studies including patients with various pelvic inflammatory/ pain syndromes and rodent models of visceral inflammation correlate elevated chemokine/

receptor expression with inflammation. Chemokine expressional changes have already been demonstrated in the urinary bladder with CYP-induced cystitis (Yuridullah et al., 2006; Sakthivel et al., 2008; Vera et al., 2008). Additionally, elevated chemokines levels have been detected in the seminal plasma and peripheral immune cells of patients with pelvic inflammatory/ pain syndromes such as ulcerative colitis, chronic prostatitis, chronic pelvic pain syndrome and IC/BPS (Penna et al., 2007; Mikami et al., 2008; Sakthivel et al., 2008). Blockade of the chemokine CXCL10 reduces symptoms of CYP-induced bladder inflammation such as hyperplasia and infiltration of immune cells (Sakthivel et al., 2008). Despite the numerous reports correlating chemokine expression with visceral inflammation, few studies have examined functional roles for chemokine signaling in urinary bladder function. We hypothesized that expression of CXCL12/CXCR4 would increase with CYP-induced bladder inflammation and blockade of CXCR4 receptor signaling with AMD3100, a specific CXCR4 antagonist, would reduce CYP-induced bladder hyperreflexia. These hypotheses encompass aims 1 and 2 and are described in chapter 2.

AKT is a putative mediator of cellular survival, however recent studies implicate the signaling molecule in visceral inflammation, pain processes (Sun et al., 2006; Sun et al., 2007; Xu et al., 2007; Shi et al., 2009) and plasticity associated with long term effects (i.e. LTP, central sensitization). Phosphorylation of AKT increases in the spinal cord DH after chemically-induced colitis and increases in the urinary bladder with CYP-induced bladder inflammation (Chung et al., 2010). The functional contribution of pAKT to micturition had not been evaluated previously. Therefore, aim 3 of this dissertation

examined activation of AKT in the urinary bladder with CYP-induced cystitis of varying duration; in addition, aim 3 addresses the functional contribution of pAKT to both inflamed and no inflamed micturition via the use of inhibitors of AKT phosphorylation, AKT Inhibitor IV and deguelin. Details of these experiments and results are described in chapter 3.

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Chapter 2: Expression and Function of CXCL12/CXCR4 in Rat Urinary Bladder with Cyclophosphamide-induced Cystitis

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Abstract

Chemokines, otherwise known as chemotactic cytokines, are pro-inflammatory mediators of the immune response and have been implicated in altered sensory processing, hyperalgesia and central sensitization following tissue injury or inflammation. To address the role of CXCL12/CXCR4 signaling in normal micturition and inflammation-induced bladder hyperreflexia, bladder inflammation in adult female Wistar rats (175-250g) was induced by injecting CYP intraperitoneally at acute (150 mg/kg; 4 hr), intermediate (150 mg/kg; 48 hr) and chronic (75 mg/kg; every third day for 10 days) time points. CXCL12, and its receptor, CXCR4, were examined in the whole urinary bladder of control and CYP-treated rats using enzyme-linked immunosorbent assays (ELISAs), quantitative PCR (qRT-PCR) and immunostaining techniques. ELISAs, qRT-PCR and immunostaining experiments revealed a significant ($p \leq 0.01$) increase in CXCL12 and CXCR4 expression in the whole urinary bladder, and particularly in the urothelium, with CYP treatment. The functional role of CXCL12/CXCR4 signaling in micturition was evaluated using conscious cystometry with continuous instillation of saline and CXCR4 receptor antagonist (AMD3100; 5 μ M) administration in control and CYP (48 hr)-treated rats. Receptor blockade of CXCR4 using AMD3100 increased bladder capacity in control (no CYP) rats and reduced CYP-induced bladder hyperexcitability as demonstrated by significant ($p \leq 0.01$) increases in intercontraction interval, bladder capacity and void volume. These results suggest a role for CXCL12/CXCR4 signaling in both normal micturition and with bladder hyperreflexia following bladder inflammation.

Introduction

Chemokines, chemotactic cytokines, are a large family of structurally and functionally related agents that are well known mediators of immune responses and inflammatory processes (53, 57). More recently however, studies suggest roles for chemokine signaling in sensory and nociceptive processes. Under physiologic conditions, with the exception of CXCL12 (73), neuronal chemokine expression is sparse; however, following nerve injury or inflammation, expression of chemokines and associated receptors increases significantly in sensory neurons, glia, macrophages, infiltrating T-cells, spinal cord and dorsal root ganglia (6, 7, 41, 53, 65, 67, 74). Additionally, elevated neuronal chemokine expression or exogenous application correlates with the maintenance of persistent pain while blockade of chemokine signaling attenuates pain behavior (6, 46, 49).

Interstitial cystitis/ painful bladder syndrome (IC/ PBS) is a chronic bladder syndrome with symptoms of urgency, frequency, nocturia, suprapubic and pelvic pain (51). IC/PBS patients have a lower threshold for sensing bladder volume and often experience pain at normal bladder pressures which suggests altered sensory processing within the urinary tract and/or its innervation (20, 47). Although some theories exist as to the underlying mechanisms of this syndrome, the etiology remains largely unclear (18, 30, 32, 50, 51, 55). However, the majority of bladder biopsies from IC/PBS patients reveal some degree of inflammatory infiltration (56) suggesting that inflammatory mediators, including chemokines may contribute to inflammatory-induced changes including urinary bladder and sensory dysfunction.

Potential roles chemokine/receptor signaling in the urinary bladder are emerging. Previous studies have shown that the expression of chemokines and/or receptors is increased following cyclophosphamide (CYP) – induced bladder inflammation (54, 66, 78). Recent studies demonstrated increased serum expression of chemokines in IC/PBS patients (54). Furthermore, in chemically-induced colitis CXCL12 is increased in the colon, and its main receptor, CXCR4, is increased in peripheral T-cells and leukocytes (45). More recently, CXCR7 has been demonstrated as a second receptor binding to CXCL12 (79, 81). Blockade of CXCR4 reduces colonic inflammation (45) and attenuates somatic sensitivity (7). CYP causes bladder hyperreflexia and induces neurochemical (68, 69, 71, 83), organizational (69, 72) and electrophysiological (31, 77) changes within the bladder and bladder afferent neurons. Potential mediators of urinary bladder inflammation are numerous: cytokines (19, 40, 43), chemokines (54, 66, 78), neuropeptides (12, 68), neuroactive compounds (11) and growth factors (70, 76, 82).

The present study addresses CXCL12/CXCR4 expression and regulation in urinary bladder and micturition reflexes using a CYP-induced model of bladder inflammation. We determined 1) expression and regulation of CXCL12 and its receptor CXCR4 in the urinary bladder using immunohistochemistry, quantitative PCR (qRT-PCR) and enzyme-linked immunosorbent assays (ELISA) with CYP-induced bladder inflammation of varying duration and 2) the effects of CXCR4 blockade with AMD3100, a compound known to specifically block CXCR4 (26, 44), on micturition reflexes using conscious cystometry with continuous intravesical instillation of saline in both CYP-

treated and control (non-inflamed) female rats. Parts of these studies have been published in abstract form (3).

Materials and Methods

Animals

Adult female Wistar rats (150-250g), purchased from Charles River Canada (St. Constant, PQ, Canada), were housed two per cage and maintained in standard laboratory conditions with free access to food and water. The University of Vermont Institutional Animal Care and Use Committee approved all animal use procedures (protocol 08-085). Animal experimentation was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Induction of CYP-induced cystitis

Rats were anesthetized with isoflurane (2%) and received intraperitoneal (i.p.) injection(s) of CYP (Sigma Aldrich, St. Louis, MO) to produce urinary bladder inflammation. To induce chronic bladder inflammation, CYP was injected (75 mg/kg; i.p.) every third day for 10 days with euthanasia occurring on the tenth day (16, 37, 38). To induce acute bladder inflammation, CYP was injected (150 mg/kg; i.p.) with euthanasia occurring 4 or 48 hours after injection (16, 37, 38). Control rats received no treatment. For conscious cystometry studies, rats received CYP as described with bladder function testing occurring 48 hours after injection.

Preparation of tissue samples for enzyme-linked immunosorbent assays (ELISAs)

Rats from all experimental groups (control, 4 hr, 48 hr and chronic; n = 4) were euthanized with isoflurane (4%), a thoracotomy performed and the urinary bladder was harvested. Individual bladders were immediately weighed and solubilized in tissue protein extraction reagent (1 g tissue/ 20 ml; Pierce Biotechnology, Woburn, MA) and treated with Complete protease inhibitor cocktail tablets (Roche Indianapolis, IN) (14, 78). Tissue was homogenized using a Polytron homogenizer and centrifuged (10,000 rpm for 10 min). The resulting supernatant was used for CXCL12 protein quantification. Total protein was determined using the Coomassie Plus Protein Assay Reagent Kit (Pierce). CXCL12 was quantified using standard 96-well ELISA plates (R&D Systems, Minneapolis, MN) according to the manufacturer's recommendations.

ELISAs for CXCL12 in urinary bladder

Microtiter plates (R&D Systems) were coated with anti-CXCL12 antibody. Sample and standard solutions were run in duplicate. Horseradish peroxidase-streptavidin conjugate was used to detect the antibody complex. Tetramethylbenzidine was the substrate, and the enzyme activity was measured by the change in optical density. The standards generated produced a linear curve. The absorbance values of standards and samples were corrected by the subtraction of the background value (absorbance due to nonspecific binding). Samples were not diluted and no samples fell below the detection limits of the assays.

Immunohistochemical localization of CXCL12 and CXCR4 in the urothelium

The bladders were rapidly dissected, placed in 4% paraformaldehyde followed by overnight incubation in 30% sucrose in 0.1M phosphate buffered-saline (PBS) for

cryoprotection. Tissue was frozen in optimal cutting temperature (O.C.T.) compound, sectioned (20 μ m) on a freezing cryostat and mounted directly onto gelled (0.5%) microscope slides (14, 16). Sections were incubated overnight at room temperature in rabbit anti-CXCL12 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-CXCR4 (1:2000; Sigma Aldrich, St. Louis, MO). Antibodies were diluted in 1% goat serum and 0.1 M phosphate buffer. After overnight incubation, sections were washed (3 x 10 min) with 0.1 M PBS (pH 7.4). Sections were then incubated with Cy3-conjugated, species-specific secondary antibodies for 2 hours at room temperature followed by washes (3 x 10 min) with PBS and coverslipping with Citifluor (Citifluor Ltd., London). Control tissue sections were incubated with 1% goat serum and 0.1 M phosphate buffer alone (no primary antibody), followed by normal washing and incubation with secondary antibodies in order to evaluate background staining levels. In the absence of primary antibody, no positive immunostaining was observed.

Visualization and semi-quantitative analysis of CXCL12 and CXCR4 in urothelium

CXCL12- and CXCR4-IR staining in bladder sections was visualized and images were captured using an Olympus fluorescence photomicroscope. The filter was set with an excitation range of 560-569 nm and emission range of 610-655 nm for visualization of Cy3. Images were captured, acquired in tiff format and imported into Meta Morph image analysis software (version 4.5r4; University Imaging, Downingtown, PA) (14, 16). The free hand drawing tool was used to select the urothelium and the urothelium was measured in total pixels area (14, 16). A threshold encompassing an intensity range of 100-250 grayscale values was applied to the region of interest in the least brightly stained

condition first. The threshold was adjusted for each experimental series using concomitantly processed negative controls as a guide for setting background fluorescence. The same threshold was subsequently used for all images.

Immunoreactivity was considered to be positive only when the staining for the marker of interest (CXCL12 or CXCR4) exceeded the established threshold. Percent marker expression above threshold in the total area selected was calculated.

Assessment of immunohistochemical staining in urinary bladder regions

Immunohistochemistry and subsequent evaluation of CXCL12- and CXCR4-IR in bladder sections or whole mount preparations were performed on control and experimental tissues simultaneously to reduce the incidence of staining variation that can occur between tissues processed on different days. Staining in experimental tissue was compared with that in experiment-matched negative controls. Urinary bladder sections or whole mounts exhibiting immunoreactivity that was greater than background level in experiment-matched negative controls were considered positively stained. Control sections incubated in the absence of primary or secondary antibody were also processed and evaluated for specificity or background staining levels. In the absence of primary antibody, no positive immunostaining was observed. Preabsorption of CXCL12 or CXCR4 antisera with appropriate immunogen (CXCL12: 1µg/ ml; CXCR4: 1-3 µg/ ml) reduced staining to background levels.

During pilot studies, additional antibodies for both CXCL12 (1:100; R&D Systems, Minneapolis, MN) and CXCR4 (1:1000; MBL, Woburn, MA) were evaluated to determine if staining was comparable in urinary bladder with CYP-induced cystitis.

The pattern of staining observed for both CXCL12 and both CXCR4 antibodies in urinary bladder after CYP treatment was consistent.

Immunohistochemical localization of CXCL12 and CXCR4 in suburothelial nerve plexus in urinary bladder whole mounts

The urinary bladder was dissected rapidly and placed into oxygenated (95% O₂ and 5% CO₂) physiologic saline solution (119.0 mmol NaCl, 4.7 mmol KCl, 24.0 mmol NaHCO₃, 1.2 mmol KH₂PO₄, 1.2 mmol MgSO₄*7H₂O, 11.0 mmol Glucose) (14, 37, 78). Starting at the urethra, a midline incision was made through the bladder and it was pinned flat onto a Sylgard-coated dish, maximally stretched, and then fixed in 2% paraformaldehyde + 0.2% picric acid for 1.5 hours. After fixation, the urothelium was separated from the detrusor layer using fine-tip forceps, iris scissors and a dissecting microscope as previously described (14, 37, 78). Notches were made in the region of the bladder neck in order to track orientation and assess regional immunoreactivity of the bladder.

Urothelium and bladder musculature were processed for CXCL12- and CXCR4-IR as described previously. In some whole mounts processed for CXCL12- and CXCR4-IR, nerve fibers in the suburothelial nerve plexus were also stained with the pan-neuronal marker protein gene product (PGP9.5, 1: 3000; AbD Serotec, Raleigh, NC) and stained with Cy2-conjugated species-specific secondary antibodies.

Visualization of CXCL12- and CXCR4-IR in suburothelial plexus in bladder whole mounts

Whole mount tissue from control and experimental groups were examined with a multiband filter set for simultaneous visualization of the Cy3 and Cy2 fluorophores. Cy2 was viewed using a filter with an excitation range of 447-501 nm and an emission range from 510-540 nm. Double-labeling in whole mount preparations of urothelium and detrusor was assessed by confocal laser scanning microscopy (Bio-Rad Laboratories, CA and Zeiss LSM 510 Meta, Carl Zeiss, Inc., NY; 38). For each z-axis interval (1-2 μ m), tissue sections were scanned twice using argon lasers with specific excitation wavelengths and sequential images were captured for computer-generated overlay and analysis.

CXCL12 and CXCR4 transcript expression in urinary bladder using Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Bladders were harvested and the urothelium was separated from the detrusor as described above. Total RNA was extracted using the STAT-60 total RNA/mRNA isolation reagent (Tel-Test 'B', Friendswood, TX, USA) as previously described (22, 38). One to 2 mg of RNA per sample was used to synthesize complementary DNA using SuperScript II reverse transcriptase and random hexamer primers with the SuperScript II Preamplification System (Invitrogen, Carlsbad, CA, USA) in a 20 μ l final reaction volume. Amplification of cDNA was performed using oligonucleotide primers specific for CXCL12, CXCR4, CXCR7, L32 and 18S. Oligonucleotide primer sequences were as follows: TGCATCAGTGACGGTAAGCCA (upper, CXCL12 130U21), ATCCACTTTAATTCGGGTCAA (lower, CXCL12 296L22),

TCCTGCCCACCATCTATTTTATC (upper, CXCR4 185U23),
ATGATATGCACAGCCTTACAT (lower, CXCR4 390L21),
ACGTGCAAGATCACACACCTCAT (upper, rCXCR7 346U23) and
GATCTTCCGGCTGCTGTGTTTCT (lower, rCXCR7 737L23).

The quantitative PCR standards for all transcripts were prepared with the amplified CXCL12, CXCR4, CXCR7, L32 and 18S cDNA products ligated directly into pCR2.1 TOPO vector using the TOPO TA cloning kit (Invitrogen). The nucleotide sequences of the inserts were verified by automated fluorescent dideoxy dye terminator sequencing (Vermont Cancer Center DNA Analysis Facility). To estimate the relative expression of the receptor transcripts, 10-fold serial dilutions of stock plasmids were prepared as quantitative standards. The range of standard concentrations was determined empirically.

Real-time quantitative PCR was performed using SYBR Green I detection (22, 38). Complementary DNA templates, diluted 10-fold to minimize the inhibitory effects of the reverse transcription reaction components, were assayed using HotStart-IT SYBR Green qPCR Master Mix (USB Corporation, Cleveland, Ohio) containing 5 mM MgCl₂, 0.4 mM dATP, dGTP, dCTP and dTTP, HotStart-IT Taq DNA polymerase and 300 nM of each primer in a final 25-ml reaction volume. The real-time quantitative PCR was performed on an Applied Biosystems 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) (22, 38) using the following standard conditions: (i) 94 °C for 2 min; (ii) amplification over 40 cycles at 94 °C for 15 s and 58 to 60 °C depending on primer set for 30 s.

The amplified product from these amplification parameters was subjected to SYBR Green I melting analysis by ramping the temperature of the reaction samples from 60 °C to 95 °C. A single DNA melting profile was observed under these dissociation assay conditions demonstrating amplification of a single unique product free of primer dimers or other anomalous products.

For data analyses, a standard curve was constructed by amplification of serially diluted plasmids containing the target sequence. Data were analyzed at the termination of each assay using the Sequence Detection Software version 1.3.1 (Applied Biosystems, Norwalk, CT). In standard assays, default baseline settings were selected. The increase in SYBR Green I fluorescence intensity (ΔR_n) was plotted as a function of cycle number and the threshold cycle was determined by the software as the amplification cycle at which the ΔR_n first intersects the established baseline. All data are expressed as the relative quantity of the gene of interest normalized to the relative quantity of housekeeping gene. Control samples were set equal to 100%.

Intravesical Catheter Implant

A lower midline abdominal incision was performed under general anesthesia with 2-3% isoflurane using aseptic techniques (13, 29, 39). The end of polyethylene tubing (PE-50, Clay Adams, Parsippany, NJ) was flared with heat and inserted into the dome of bladder and secured in place with a 6-0 nylon purse-string suture (13, 29, 39). The distal end of the tubing was sealed and tunneled subcutaneously to the back of the neck where it was externalized, out of the animal's reach (13, 29, 39). Rats received buprenorphine (0.05 mg/kg, s.c.) starting at the time of surgery and then every 8-12 hours postoperatively for a

total of 4 doses. Animals were maintained for 72 hours after surgery before conscious cystometry was initiated to ensure complete recovery.

Conscious cystometry with continuous intravesical infusion of saline and CXCR4 blockade

The effects of CXCR4 blockade on bladder function in control (no inflammation) and CYP-treated rats (48 hours) were evaluated by pharmacological receptor blockade with infusion of AMD3100 (5 μ M; Sigma Aldrich, St. Louis, MO), known to specifically block CXCR4 (26, 44) using conscious cystometry and continuous infusion of intravesical saline. During cystometry, unrestrained and conscious rats were placed in a recording cage over a scale and pan in order to collect and measure voided urine. To elicit repetitive bladder contractions, room temperature saline was infused at a constant rate (10 ml/hr). At least six reproducible micturition cycles were recorded after an initial stabilization period (25 - 30 minutes). Intravesical pressure changes were recorded using a Small Animal Cystometry System (Med Associates, Inc., St. Albans, VT) (13, 38). Baseline resting pressure, pressure threshold for voiding, maximal voiding pressure and intercontraction interval were measured prior to AMD3100 instillation. Non-voiding bladder contractions (NVCs), defined as rhythmic intravesical pressure increases 7 cm H₂O above baseline without the release of fluid from the urethra, were also determined per voiding cycle. Bladder capacity was measured as the amount of saline infused into the bladder at the time when micturition commenced (10, 27).

To evaluate the effects of CXCR4 blockade on bladder function, immediately following the baseline recordings, rats were anesthetized (1-2% isoflurane) and

AMD3100 (5 μ M), known to specifically block CXCR4 (25, 41), was intravesically infused for 30 minutes. The concentration selected for evaluation was based upon published studies (35). Prior to intravesical drug infusion, the bladder was manually emptied via the Credé maneuver. Bladders were then infused with approximately 1 ml (less than bladder capacity to not elicit a bladder contraction and expulsion of instillate) of AMD3100 (5 μ M) according to prior published studies (13, 39). Rats remained anesthetized (1-2% isoflurane) to subdue the micturition reflex and prevent expulsion of the CXCR4 receptor antagonist from the bladder. Immediately following drug treatment, bladder function testing was repeated. To avoid potential variation resulting from circadian rhythms, experiments were conducted at similar times of the day (17). At the conclusion of the study, rats were euthanized as described above. To determine if the experimental design of the studies (i.e., baseline bladder function recordings, anesthesia and intravesical drug instillation, additional bladder function recordings) could affect cystometric recordings, additional rats (control, no inflammation and 48 hour CYP-treated) underwent baseline recordings, anesthesia and intravesical saline instillation and additional cystometric analyses. These studies with identical experimental design and intravesical saline instillation revealed no changes in cystometric parameters analyzed (data not shown).

Exclusion Criteria

Rats were removed from the study when adverse events occurred that included: $\geq 20\%$ reduction in body weight post surgery, a significant postoperative event, lethargy, pain, or distress not relieved by our IACUC-approved regimen of postoperative analgesics or

hematuria in control rodents (13, 39). In the present study, no rats were excluded from the study or from analysis due to any of these exclusion criteria. In addition, behavioral movements such as grooming, standing, walking, and defecation rendered bladder pressure recordings during these events unusable.

Materials

AMD3100 was purchased from Sigma Aldrich (St. Louis, MO), prepared as concentrated stock solutions, aliquoted and stored at -20 °C until usage. Aliquots were diluted with saline to achieve final concentration.

Figure Preparation

Digital images were obtained using a charge-coupled device camera (MagnaFire SP, Optronics; Optical Analysis) and LG-3 frame grabber attached to an Olympus microscope (Optical Analysis, Nashua, NH). Exposure times were held constant when acquiring images from control and experimental animals processed and analyzed on the same day. Images were imported into Adobe Photoshop 8.0 (Adobe Systems, San Jose, CA) where groups of images were assembled and labeled.

Statistical Analyses

All values represent mean \pm SEM. Data were compared with analysis of variance (ANOVA) or repeated measures ANOVA, where appropriate. When F ratios exceeded the critical value ($P \leq 0.05$), the Newman-Keul's or Dunnett's post hoc tests were used to compare group means.

Results

CXCL12 Protein Expression in the Rat Urinary Bladder with CYP-Induced Cystitis

CXCL12 protein expression in the whole urinary bladder increased significantly ($p \leq 0.01$) after 48 hr (1.8-fold) and chronic CYP treatment (1.9-fold; Fig. 1) as determined with ELISAs. CXCL12 bladder expression with 48 hr CYP treatment did not differ from chronic CYP treatment. No change in CXCL12 urinary bladder expression was observed with 4 hr CYP treatment.

CXCL12, CXCR4 and CXCR7 mRNA Expression in the Urinary Bladder of Rats with and without CYP-Induced Cystitis

Quantitative PCR demonstrated a significant ($p \leq 0.01$) increase in CXCL12 mRNA in both the urothelium and detrusor with 48 hr and chronic CYP treatment but not 4 hr CYP treatment (Fig. 2A, B). CXCR4 mRNA was increased significantly ($p \leq 0.05$) with 48 hr CYP treatment in both the urothelium and detrusor (Fig. 2C, D). No change in CXCR4 mRNA expression was observed at 4 hr or chronic CYP-treatment time points (Fig. 2C, D). CXCR7 increased significantly ($p \leq 0.05$) after chronic CYP -treatment in both the urothelium and the detrusor (Fig. 3A, B). No change in CXCR7 mRNA expression was observed at the 4 hr or 48 hr CYP-treatment time points (Fig. 3A, B).

CXCL12 and CXCR4 Immunoreactivity (IR) in Urinary Bladder with CYP-induced Cystitis

Urinary bladder sections Basal CXCL12-IR expression was present in the urothelium in all urothelial layers (basal, intermediate and apical), lamina propria and more diffusely in

the detrusor in control (no inflammation) urinary bladder (Fig. 4A, C). When examining CXCL12-IR following CYP treatment, we focused on the 48 hr time point because it is the earliest CYP treatment where we observed an increase in CXCL12 mRNA and protein expression in the whole urinary bladder. Additionally, neither CXCL12 mRNA nor the protein expression levels differed from the chronic CYP treatment time point. Following 48 hr CYP-treatment, CXCL12-IR expression was robust in the urothelium and lamina propria (Fig. 4B, D). We focused our analyses of CXCL12 expression in the urothelium because expression was most dramatically upregulated in the urothelium with CYP-treatment. Urothelial CXCL12-IR was significantly ($p \leq 0.01$) increased (2.4 -fold) with 48 hour CYP-treatment (Fig. 5A). In control (no inflammation) bladders, CXCR4-IR expression was virtually absent in the lamina propria and detrusor, but basal urothelial CXCR4-IR expression was present (Fig. 4E, G). Following 48 hr CYP treatment, urothelial CXCR4-IR increased significantly ($p \leq 0.01$; 3.5 -fold; Fig. 4F, H; Fig 5B).

Bladder whole mounts CXCL12- or CXCR4-IR in the suburothelial nerve plexus was not observed in control or CYP-treated whole mount bladder preparations. Multiple attempts ($n = 6-10$) with different species-specific secondary antibodies to visualize CXCL12- or CXCR4-IR in the suburothelial plexus were not successful (data not shown). Further, despite PGP immunostaining of the suburothelial nerve plexus, colocalization with either CXCL12- or CXCR4-IR was not observed with confocal or indirect immunofluorescence techniques (data not shown). In contrast, whole mount preparations exhibited urothelial, lamina propria and detrusor smooth muscle staining for

CXCL12 or CXCR4 consistent with staining observed in cryostat bladder sections (data not shown).

CXCR4 Blockade with Intravesical Infusion of AMD3100 using Conscious Cystometry in Rats with and without CYP-induced cystitis

Control (no inflammation). Conscious cystometry was performed in control rats prior to drug treatment to establish baseline voiding frequency, bladder capacity and void volume (Figs. 6A and 7). Following intravesical infusion of AMD3100 (5 μ M), a CXCR4 receptor antagonist, the same rats exhibited decreased voiding frequency coupled with an increase (1.3-fold) in both bladder capacity, as measured as the amount of saline infused into the bladder at the time when micturition commenced ($p \leq 0.01$; Figs. 6B and 7), and duration of intercontraction interval (ICI; $p \leq 0.01$; Figs. 6B and 7), and an increase (1.3-fold) in void volume ($p \leq 0.05$; Figs. 6B and 7). There were no changes in threshold, filling or peak micturition pressures following AMD3100 treatment in control rats (Table 1).

CYP treatment. As previously demonstrated (13, 28, 29, 39), CYP treatment (48 hr) increased void frequency and decreased bladder capacity, ICI and void volume (Figs. 8A and 9). Additionally, CYP treatment increased ($p \leq 0.01$) filling, threshold and micturition pressures (Table 1). Following intravesical infusion of AMD3100 (5 μ M), the same rats exhibited decreased voiding frequency and significantly ($p \leq 0.001$) increased bladder capacity (1.9-fold), as measured as the amount of saline infused into the bladder at the time when micturition commenced, ICI (1.9-fold) and increased void

volume (2.2-fold; Figs. 8 and 9). There were no changes in threshold, filling or peak micturition pressures following AMD3100 treatment in CYP treated (48 hr) rats (Table 1).

Non-voiding contractions (NVCs; increases in baseline pressure with an amplitude ≥ 7 cm H₂O without the expulsion of urine) occurred very infrequently in the evaluated rats with CYP treatment before or after intravesical infusion of AMD3100. Therefore, the effect of AMD3100 on NVCs was not assessed.

Discussion

These studies demonstrate several novel findings with respect to chemokine/receptor regulation and function in micturition reflexes under normal or inflamed bladder conditions in female rats. We have previously shown CYP-induced changes in urinary bladder expression of the chemokine/ receptor pair: CX3CL1/CX3CR1 (78). In the present studies we demonstrate that a second chemokine receptor pair, CXCL12/CXCR4: (1) has low basal expression in the urinary bladder; (2) increases in the urinary bladder following CYP-treatment of varying duration; (3) exhibits strong expression in the urothelium following CYP-induced cystitis; and (4) plays a role in bladder function in both normal (no CYP) and CYP-treated rats. CXCR4 blockade at the level of the urinary bladder increased bladder capacity and void volume in normal and, to a greater extent, in CYP-treated rats. To the best of our knowledge, these studies are the first to demonstrate roles for chemokine/receptor signaling in bladder function. Therefore, chemokines may represent a novel class of potential molecular targets for pharmaceutical intervention with respect to bladder inflammation.

Interstitial cystitis (IC)/ painful bladder syndrome (PBS) is a chronic bladder syndrome with symptoms of urgency, frequency, nocturia and suprapubic and pelvic pain (51). Although the etiology and pathogenesis of IC are unknown, numerous theories including infection, autoimmune disorder, toxic urinary components, deficiency in bladder wall lining and neurogenic causes have been proposed (18, 30, 32, 50, 51, 55). We have hypothesized that pain associated with IC/ PBS involves alteration of visceral sensation/ bladder sensory physiology. Altered visceral sensations from the bladder (i.e.

pain at low or moderate bladder filling) that accompany IC/ PBS may be mediated by many factors including changes in the properties of peripheral bladder afferent pathways such that bladder afferent neurons respond in an exaggerated manner to normally innocuous stimuli (allodynia). These changes may be mediated, in part, by inflammatory changes in the urinary bladder. Potential mediators of urinary bladder inflammation are numerous: cytokines (19, 40, 43), neuropeptides (12, 68), neuroactive compounds (11), growth factors (70, 76, 82) and chemokines (54, 66, 78). Increased serum levels of chemokines (e.g., CXCL9-CXCL11) in IC/PBS patients have been demonstrated (54).

Using the CYP-induced bladder inflammation model (16, 37), we aimed to characterize further the role of immune mediators, specifically chemokines, in the development and/or maintenance of altered bladder sensory physiology associated with urinary bladder inflammation. Chemokines are mediators of the innate immune response and assert protective effects by attracting leukocytes to sites of injury and by initiating extravasation and developing guiding chemotactic gradients (53, 57). Recently, mediators of the immune response, including chemokines, have received considerable attention for their potential role in heightened sensory processing and pain (53, 73). For example, exogenous administration of chemokines induces thermal hyperalgesia and mechanical allodynia (46, 49, 65). Additionally, in a model of neuropathic pain, hyperalgesia occurs concomitantly with increased chemokine signaling, through CXCR4 specifically (6). In contrast, antagonists to chemokine receptors can reduce nociceptive behavior (6, 46) and certain chemokine knockout mice fail to develop somatic sensitivity (1). Recently, Foster et al. (21) showed that hindpaw mechanical hypersensitivity

induced by unilateral injections of acidic saline into the pelvic floor muscles of rats can be ameliorated by administration of AMD3100. CYP-induced bladder inflammation in mice and rats is associated with increased, referred somatic sensitivity (13, 24, 59). Effects of CXCR4 receptor blockade on CYP-induced referred somatic sensitivity were not evaluated in this study due to experimental procedures including a laparotomy. Studies to assess the contribution of CXCR4 to CYP-induced referred somatic sensitivity would however be of interest in the future. Thus, chemokine signaling may contribute to altered somatic sensation but roles in bladder sensory physiology have not yet been considered.

Previous studies demonstrated increased bladder chemokine expression following CYP treatment (66, 78). Fractalkine (CX3CL1) and fractalkine receptor (CX3CR1) expression were increased in the urothelium with CYP-induced cystitis (48 hour and chronic). Similar to the present studies with CXCL12/CXCR4, fractalkine and fractalkine receptor were upregulated in the urothelium with little effect being observed in the detrusor smooth muscle (78). Further, neither CX3CL1/CX3CR1 nor CXCL12/CXCR4 was expressed in the suburothelial nerve plexus (78). Previous studies by Vera et al. (66) demonstrated increased CXCR4 expression and altered distribution in the urothelium of male, Sprague-Dawley rats treated chronically with CYP. However, no increased expression of CXCL12 was noted in the bladder with CYP treatment (66). Further, basal expression of CXCR4 in urothelium of control (no CYP) was greater (66) than that observed in the present study. Differences in CXCR4 and CXCL12 bladder expression between the study by Vera et al. (66) and the current study may be due to

gender and strain differences in the rats studied and/or CYP dosing differences. Gender differences may be very likely given observations that estrogen regulates chemokine/receptor expression (58). The present studies extend previous studies (54, 66, 78) by demonstrating regulation as well as function of CXCL12/CXCR4 signaling in the urinary bladder with urinary bladder inflammation induced by CYP treatment. Increases in CXCL12 and its cognate receptor CXCR4 were observed in the urinary bladder following CYP treatment using multiple techniques including qRT-PCR, ELISAs and immunostaining.

Blockade of CXCR4 with a CXCR4 receptor antagonist, AMD3100, reduced CYP-induced bladder hyperreflexia as evidenced by increased bladder capacity and decreased voiding frequency. In these studies, AMD3100 is most likely acting at the level of the urothelium for several reasons: (1) both mRNA and histologic analyses showed that the greatest expressional increase for both CXCL12 and CXCR4 following CYP treatment was in the urothelium; (2) histologically, CXCR4 had a restricted presentation being expressed only in the urothelium in both control and CYP treated bladders; (3) repeated attempts did not demonstrate CXCL12- or CXCR4-IR in the suburothelial nerve plexus. Furthermore, CXCR4 receptor blockade with AMD3100 also significantly increased bladder capacity in control animals (no inflammation) that, without CYP treatment, should have an intact urothelium. The urothelium creates an impermeable barrier and has the highest junction potential of any cell type (75). Thus, drug penetration through the urothelium to underlying structures (e.g. suburothelial plexus or detrusor smooth muscle) without use of a disrupting agent (e.g. protamine

sulfate) is unlikely (15, 39). Changes in bladder function following AMD3100 treatment in control animals should not be surprising as bladders from these animals exhibit basal urothelial CXCL12 and CXCR4 expression. The magnitude of the increase in bladder capacity observed in control animals following AMD3100 infusion was less than that observed in CYP treated rats following AMD3100 infusion. The increased magnitude of the change elicited with CXCR4 receptor blockade in CYP-treated rats may be due to: (1) increased CXCL12 and CXCR4 expression in the urinary bladder following CYP treatment and (2) a more prominent role for CXCL12/CXCR4 signaling in bladder function during inflammation.

AMD3100 is a selective CXCR4 antagonist (26, 44) however, potential actions of CXCL12 through a second receptor, CXCR7, must also be considered (23). Balabanian et al., (5) showed that CXCL12 also binds CXCR7 in T lymphocytes and that this interaction partially mediates chemotaxis. In this study, we demonstrate using qRT-PCR studies that CXCR4 as well as CXCR7 mRNA expression is regulated in the urinary bladder following CYP-induced cystitis. However, the regulation of CXCR7 mRNA expression in urinary bladder with CYP treatment differs from that observed for CXCR4. CXCR4 mRNA expression is significantly increased in urinary bladder with 48 hr CYP-treatment. In contrast, CXCR7 transcripts significantly increase in urinary bladder with chronic CYP-treatment but not with 4 hr or 48 hr CYP-treatment. Effects of AMD3100 were observed when evaluated in 48 hr CYP treated rats consistent with blockade of CXCR4. Although CXCR7 mRNA expression did not increase with 48 hr CYP treatment, basal urinary bladder expression of CXCR7 could be sufficient to contribute to

the observed changes in bladder function. Although AMD3100 is a selective CXCR4 antagonist (26, 44), AMD3100 can act as a substantially weaker CXCR7 antagonist (42, 79). In cultured cell lines, AMD3100 did not significantly affect CXCL12/CXCR7 interactions at high concentrations although AMD3100 did selectively block binding of CXCL12/CXCR4 (42). Further, a recent study also suggests that AMD3100 is a CXCR7 ligand with agonist properties when used at high concentrations (100-1000 μ M) (36). Thus, AMD3100 may have opposite effects on the two receptors, CXCR4 and CXCR7, depending on concentrations and context being evaluated (36, 79). In the current study, AMD3100 was used below the concentration ranges documented to result in agonist effects mediated through the CXCR7 receptor and it is clear that AMD3100 is more effective at blocking binding of CXCL12/CXCR4 (42). However, studies of the roles of CXCL12/CXCR4 and/or CXCL12/CXCR7 in the urinary bladder of control rats and those with urinary bladder inflammation are novel, not previously evaluated. Thus, potential effects of CXCR7 receptor blockade cannot be ruled out and it is a possibility that the overall changes in bladder function observed with AMD3100 intravesical administration represent effects, perhaps opposite (36) or those involving cross-talk (25) between CXCR4 and CXCR7. Future studies using high-affinity and selective CXCR7 ligands such as CCX771 (79) should be considered when defining the role of CXCR7 in bladder function.

To our knowledge, this is the first time the CXCR4 receptor antagonist, AMD3100, has been intravesically infused, thus we needed to determine working dilutions empirically. Based on stromal cell adherence experiments, a concentration of 50

μM was attempted first in pilot studies (4). Whereas this concentration (50 μM) of AMD3100 induced a similar increase in bladder capacity, an undesirable decrease in peak micturition pressure was also observed. Based on a concentration used in cultured epithelial cells (35) we then used 5 μM AMD3100 and observed an increase in bladder capacity without changes in pressure; therefore, 5 μM AMD3100 was used throughout the experiments in this study. This dose may not be optimal as there is no literature precedent for intravesical instillation of AMD3100. The effects on bladder function were observed in control and CYP-treated rats treated with this AMD3100 dose; the effects were only observed on intercontraction interval and bladder capacity - not on bladder pressures. The absence of broad effects on all cystometric parameters suggests specificity in AMD3100 action at the dose used in this study. Lack of effects on micturition pressures (i.e., baseline and peak micturition) in control and CYP-treated rats also suggests little or no effects on urethral outlet resistance.

Recently, the functional contribution of the urothelial lining of the urinary bladder has advanced beyond the view of the urothelium as a passive barrier to that of an active sensor with a potential signaling (i.e. sensory) role especially in the context of urinary bladder inflammation (8). Urothelial cells share a number of similarities with sensory neurons and the urothelium has been suggested (2, 8, 9, 61, 62, 63, 64) to have ‘neuronal-like’ properties. Urothelial cells express numerous receptors similar to those found in dorsal root ganglia (DRG) neurons such as purinergic, norepinephrine, acetylcholine, neuropeptide and protease-activated receptors, acid sensing ion channels, neurotrophin receptors, and transient receptor potential channels (2, 8, 9, 61, 62, 63, 64). Therefore it

was not surprising to observe CXCL12/CXCR4 expression in the urothelium in control or CYP-treated rats given distribution of chemokines and associated receptors in DRG (6, 7, 33, 34, 49, 52, 60, 65, 67, 74, 80). The present study adds to the growing list of similarities between urothelial cells and sensory neurons.

In the present studies, we did not observe CXCL12 or CXCR4 immunostaining in the suburothelial plexus and this was surprising especially for CXCR4 given the numerous reports of CXCR4 expression in DRG cells (6, 7, 48, 49). Although we did not observe CXCL12/CXCR4 immunostaining in the suburothelial nerve plexus in whole mounts, urothelial cells (CXCL12 and CXCR4) and detrusor smooth muscle (CXCL12; data not shown) did exhibit immunostaining, so technical limitations such as antibody penetration do not seem plausible. Given the expression of CXCL12/CXCR4 in urothelial cells and detrusor smooth muscle but not in the suburothelial plexus, the mechanism(s) of action of CXCL12 likely excludes direct effects on the suburothelial nerve plexus. Urothelial cells secrete many signaling molecules in response to distention and exhibit altered secretion with bladder inflammation (2, 8, 9). Such signaling molecules include: neurotrophins, neuropeptides, adenosine triphosphate (ATP), acetylcholine, prostanoids, nitric oxide (NO) and cytokines (2, 8, 9). Functional receptor expression coupled with chemical secretion capabilities enables the urothelium to respond to stimuli and reciprocally communicate with detrusor smooth muscle cells, suburothelial nerve plexus and/ or interstitial cells (2, 8, 9). Therefore, it is possible that CXCL12 signaling via CXCR4 expression in urothelial cells may activate downstream targets that consequently facilitate the release of other mediators. Urothelial derived

mediators such as ATP or NO may then influence the suburothelial nerve plexus to affect micturition reflex function (2, 8, 9). Future studies assessing CXCL12-induced secretion of ATP and/or NO in urothelial cells and CXCL12-mediated effects on detrusor contractility in the presence and absence of urothelium are logical future directions to determine direct and indirect effects of CXCL12/CXCR4 signaling in micturition pathways.

Conclusions

These studies demonstrate a functional role for CXCL12/CXCR4 signaling in bladder function in normal rats and those with urinary bladder inflammation induced by CYP. Chemokines and associated receptors signaling may serve as potential targets for therapeutic intervention with respect to bladder inflammation.

Grants

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Figures

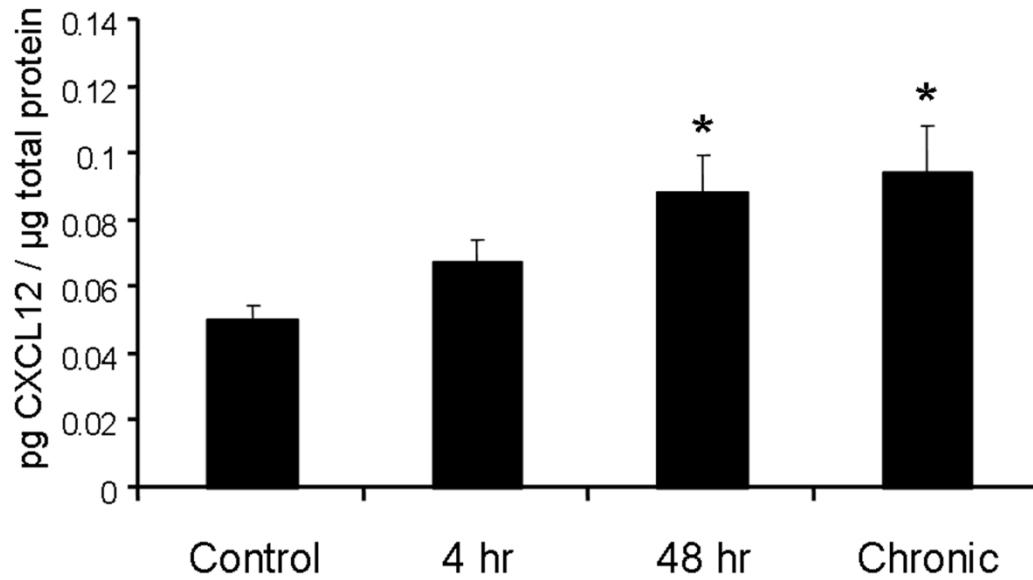


Figure 1

Figure 1: Time dependent changes in CXCL12 protein expression in whole urinary bladder following CYP treatment as determined with ELISAs. CXCL12 expression increased significantly (*, $p \leq 0.01$) at 48 hour (hr) and chronic time points compared to control urinary bladders. $n = 4$ for control and each experimental condition.

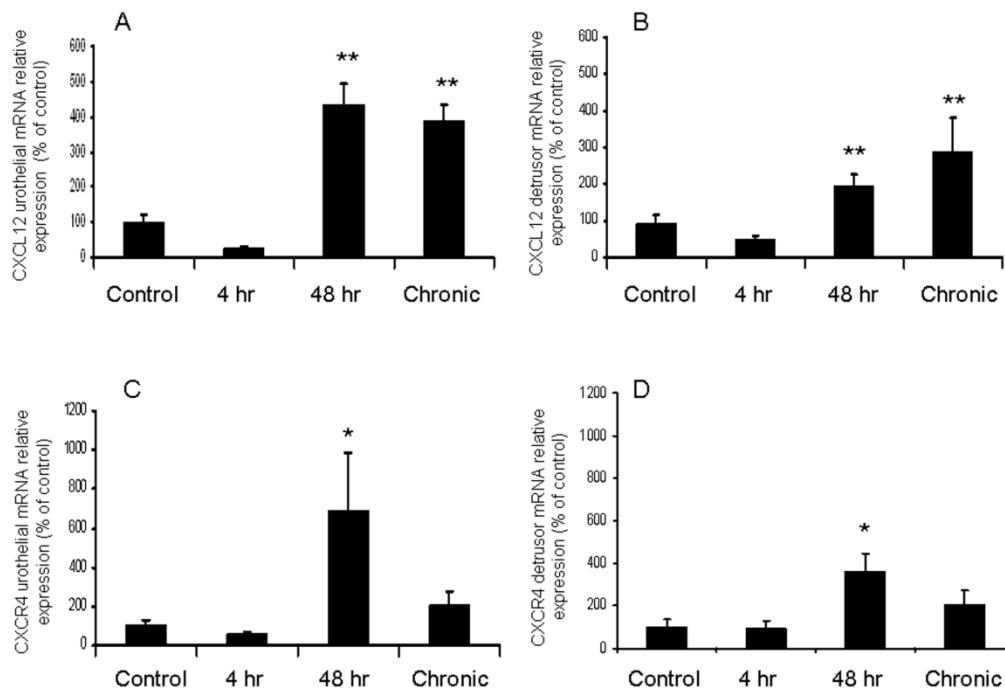


Figure 2

Figure 2: Time dependent and regional changes in CXCL12 and CXCR4 mRNA expression in urinary bladder as detected by qRT-PCR. Following 48 hr and chronic CYP treatment, CXCL12 mRNA expression was significantly increased in both the urothelium (A; **, $p \leq 0.01$) and the detrusor (B; **, $p \leq 0.01$). CXCR4 mRNA expression was significantly increased after 48 hr CYP treatment in both the urothelium (C; *, $p \leq 0.05$) and the detrusor (D; *, $p \leq 0.05$). $n = 5-7$ for each group.

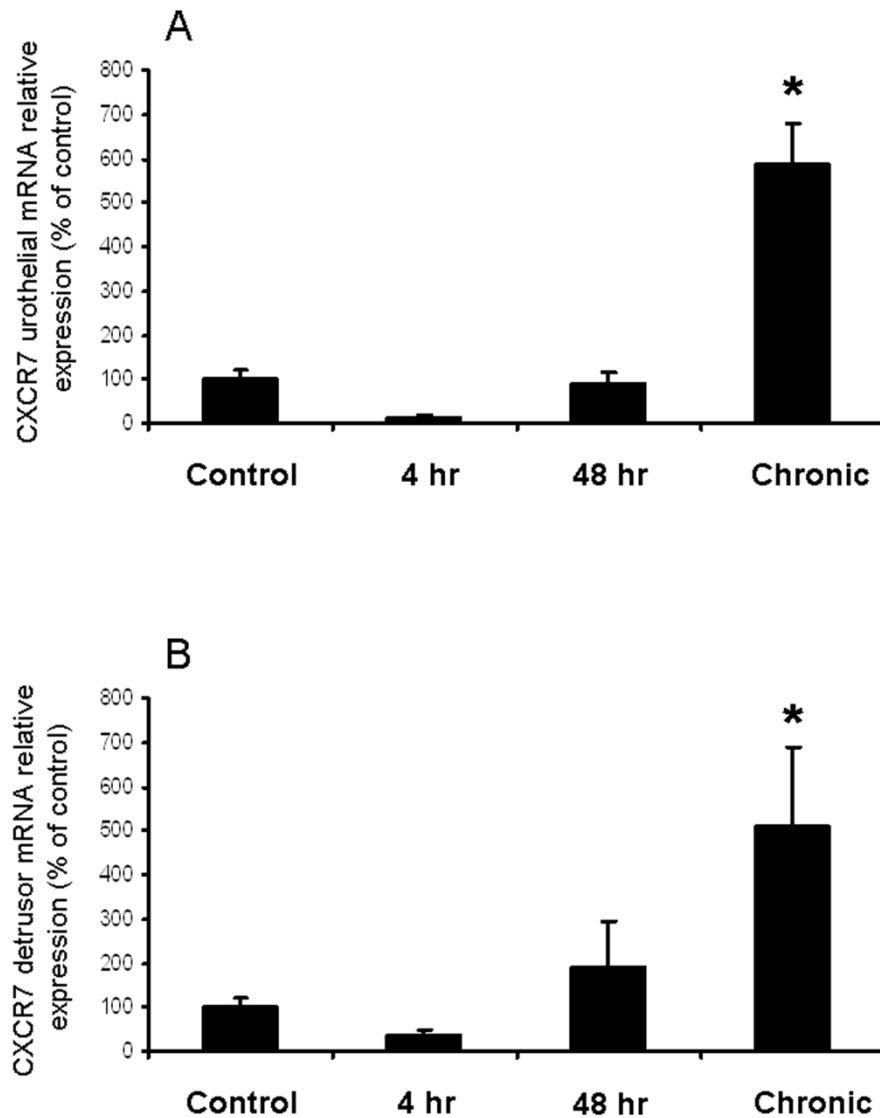


Figure 3

Figure 3: Time dependent and regional changes in CXCR7 mRNA expression in urinary bladder as detected by qRT-PCR. CXCR7 mRNA expression increased significantly in both the urothelium (A; *, $p \leq 0.05$) and the detrusor (B; *, $p \leq 0.05$) following chronic CYP-treatment but not 4 hr or 48 hr CYP-treatment (n = 5-7 for each group).

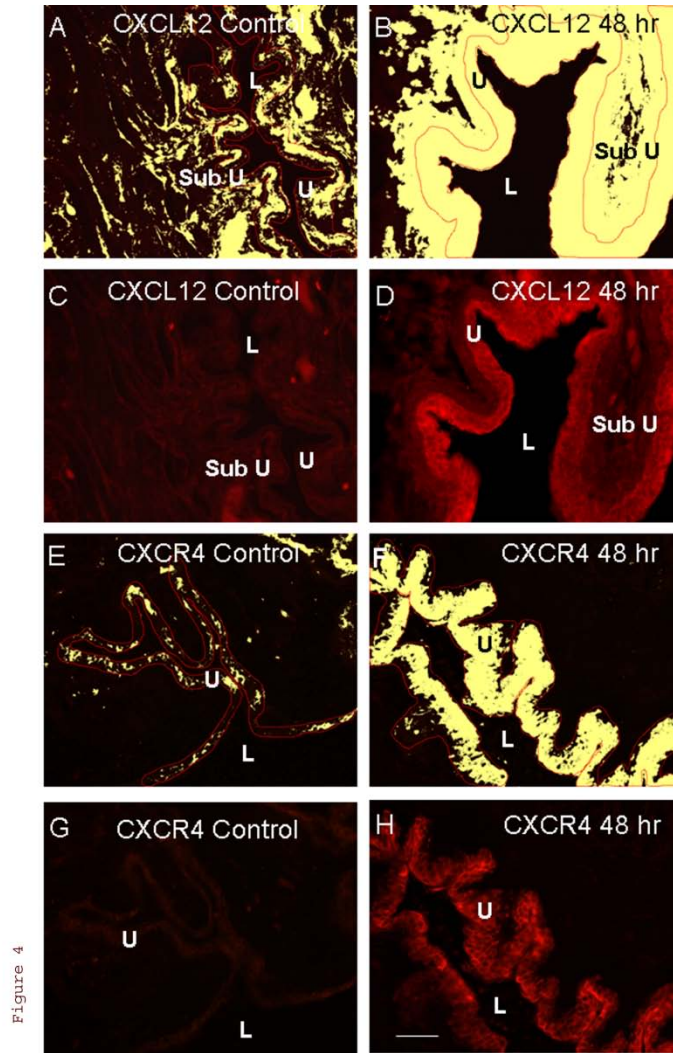


Figure 4: Regulation of CXCL12 (A-D) and CXCR4 (E-H) expression in cryostat sections of urinary bladder after CYP treatment. The urothelium was outlined in red and images were thresholded (A, B, E and F). All pixels above threshold are depicted in yellow. Corresponding fluorescence images of CXCL12 expression in control or after 48 hour (hr) CYP treatment are shown in C and D, respectively. Fluorescence images of CXCR4 expression in urinary bladder of control or after 48 hr CYP treatment are shown in G and H, respectively. (L: lumen; U: urothelium; Sub U; suburothelium). Calibration bar represents 50 μm .

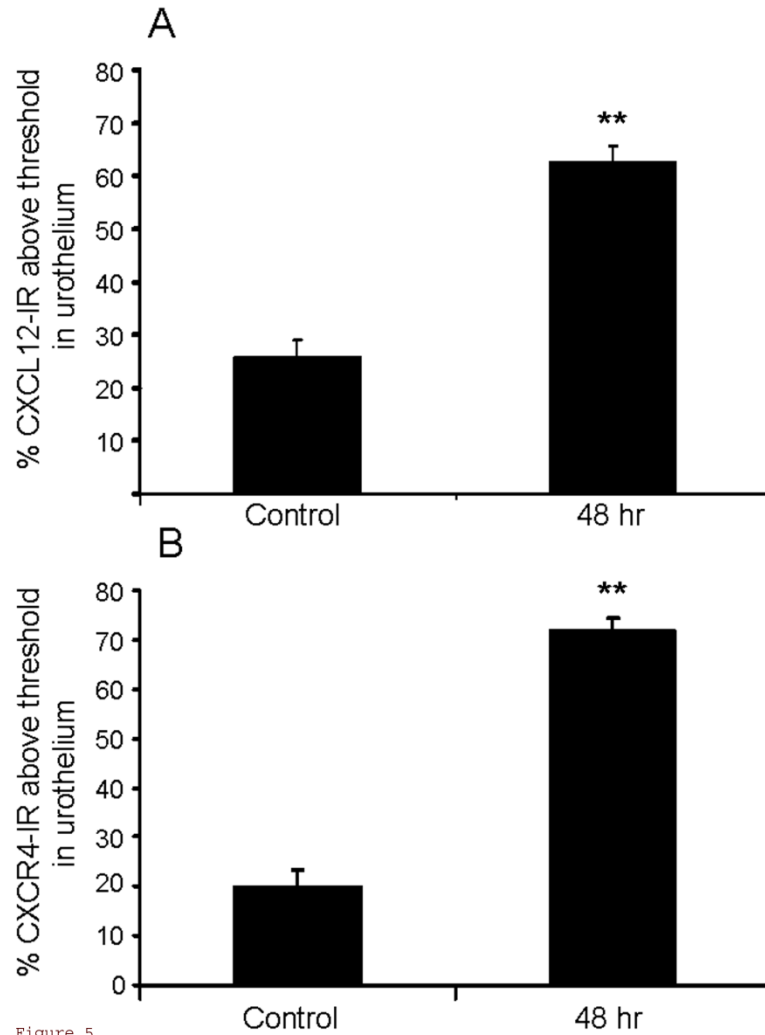


Figure 5

Figure 5: Summary histogram of CXCL12- (A) and CXCR4-IR (B) expression in the urothelium of the urinary bladder of control rats and those treated with CYP (48 hr).

Values are mean \pm SEM; (n = 3-8). **, $p \leq 0.01$.

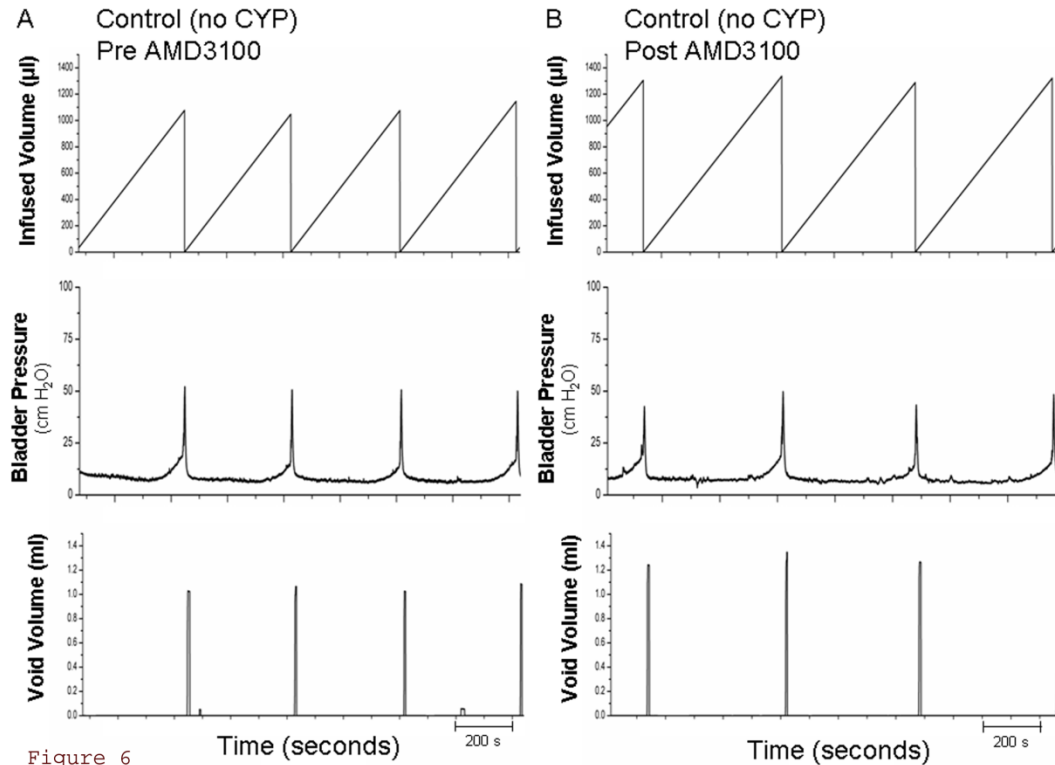


Figure 6: Representative cystometrogram recordings of effects of CXCR4 receptor blockade in control (no inflammation) rats using continuous intravesical infusion of saline. Pre AMD3100 (A) and Post AMD3100 (B) drug treatments in control rats (no CYP treatment) with continuous intravesical instillation of saline. CXCR4 receptor blockade with intravesical infusion of AMD3100 (5 μ M) increased both bladder capacity (as measured as the amount of saline infused into the bladder at the time when micturition commenced) and void volume in comparison to pre-treatment conditions (A). Bladder function recordings in A and B are recorded from the same rat.

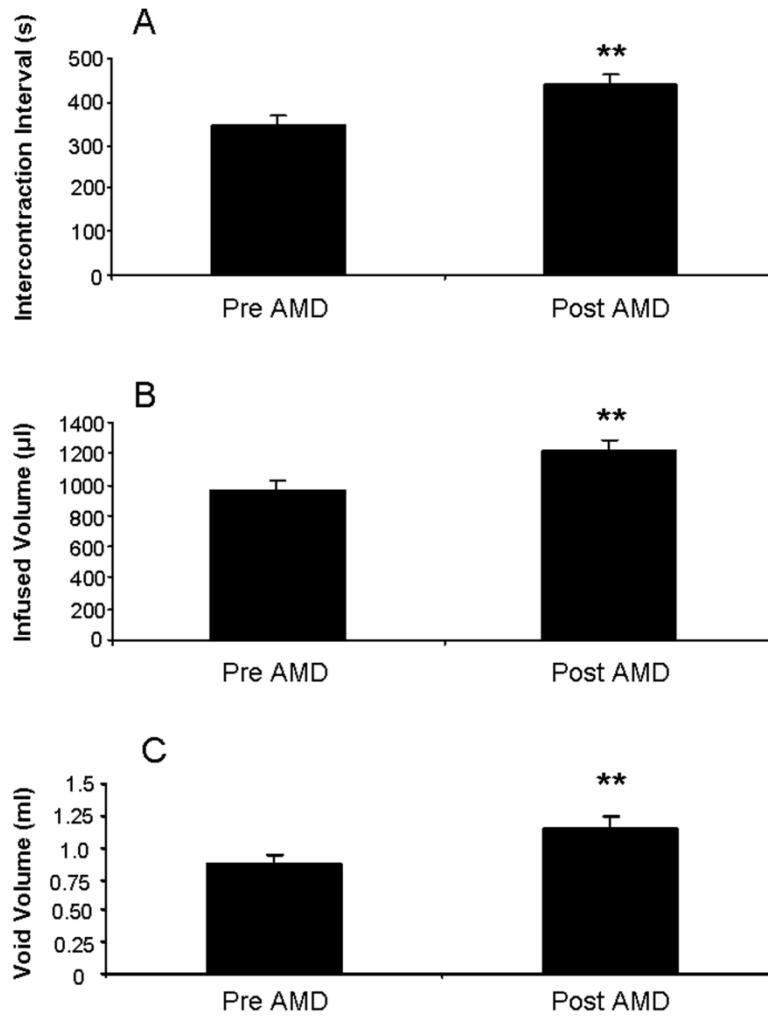


Figure 7

Figure 7: Summary histograms of the effects of CXCR4 receptor blockade with AMD3100 intravesical infusion in control rats (no CYP treatment). A: Infusion of AMD3100 (5 μ M) significantly (**, $p \leq 0.01$) increased intercontraction interval. B: Bladder capacity was also significantly (**, $p \leq 0.01$) increased along with a significantly (*, $p \leq 0.05$) increased void volume (C). Values are mean \pm SEM; (n = 4-6).

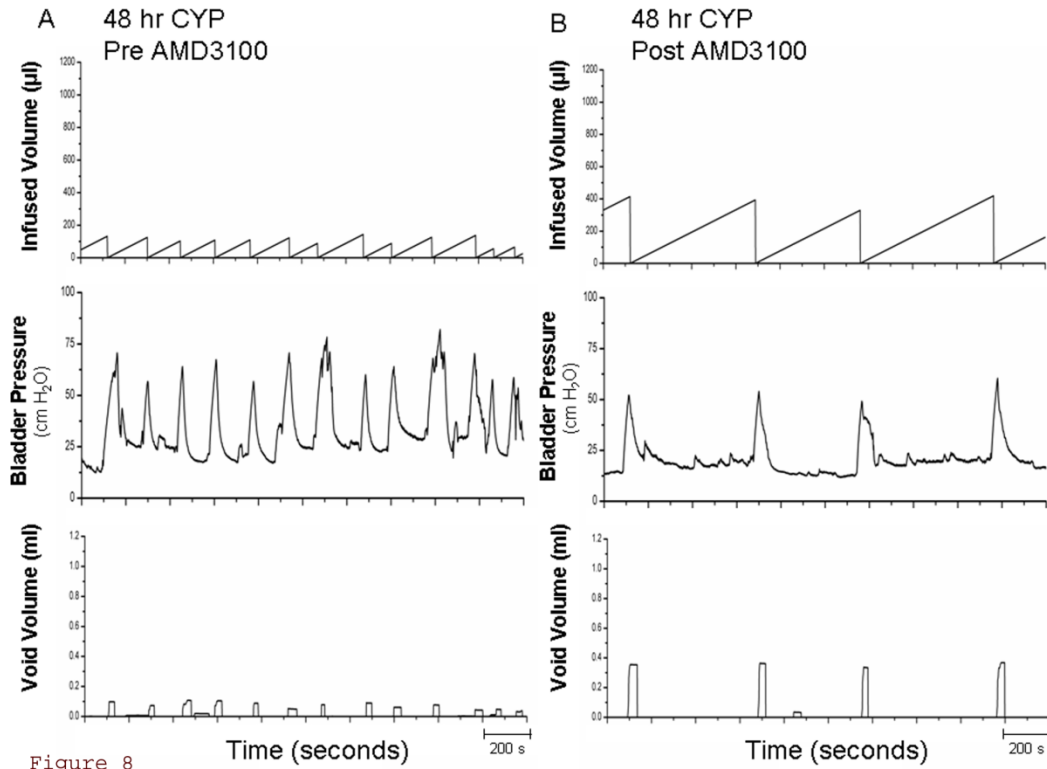


Figure 8: Representative cystometrogram recordings of effects of CXCR4 receptor blockade in CYP treated (48 hr) rats using continuous intravesical infusion of saline. Pre AMD3100 (A) and Post AMD3100 (B) drug treatments in 48 hr CYP treated rats with continuous intravesical instillation of saline. CXCR4 receptor blockade with intravesical infusion of AMD3100 (5 μ M) increased both bladder capacity (as measured as the amount of saline infused into the bladder at the time when micturition commenced) and void volume in comparison to pre-treatment conditions (A). Bladder function recordings in A and B are recorded from the same rat.

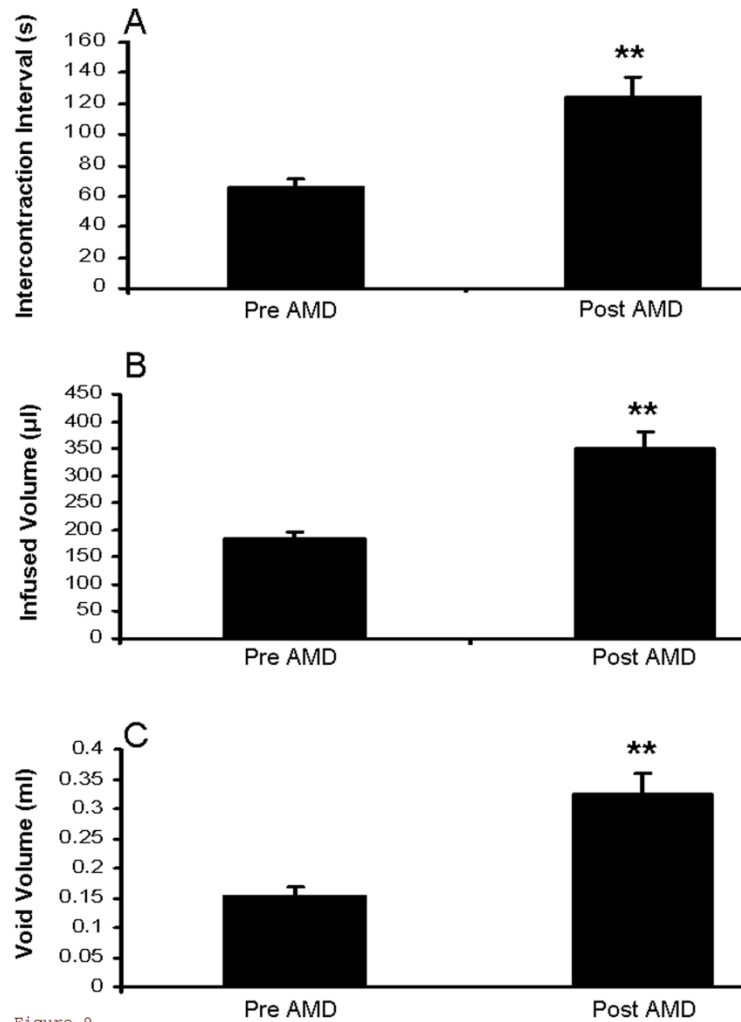


Figure 9

Figure 9: Summary histograms of effects of CXCR4 receptor blockade with AMD3100 intravesical infusion in 48 hr CYP treated rats. A: Infusion of AMD3100 (5 μ M) significantly (**, $p \leq 0.01$) increased intercontraction interval. B: Bladder capacity was also significantly (**, $p \leq 0.01$) increased along with a significantly (**, $p \leq 0.01$) increased void volume (C). Values are mean \pm SEM ($n = 6$).

Table 1: Threshold, peak micturition and filling pressures during conscious cystometry for control and 48 hr CYP treated rats both pre and post AMD3100 treatment

		Threshold Pressure (cmH ₂ O)	Micturition Pressure (cmH ₂ O)	Baseline Pressure (cmH ₂ O)
Control	Pre AMD	14.2 ± 0.7	51.7 ± 1.6	10.6 ± 0.6
	Post AMD	14.0 ± 0.9	47.7 ± 2.0	9.7 ± 0.6
48 hr CYP	Pre AMD	24.3 ± 1.1	65.4 ± 3.2	23.1 ± 1.1
	Post AMD	23.6 ± 1.2	74.5 ± 5.1	22.5 ± 1.2

Table 1: Values presented are mean ± SEM; n = 4-6 in each group.

No pressure changes were observed within experimental groups (i.e. control pre-AMD vs. post-AMD or CYP pre-AMD vs. post-AMD) following AMD3100 (5 µM) instillation; however, CYP treatment (48 hr) significantly (*, $p \leq 0.05$) increased threshold, peak micturition and filling pressures when compared to control rats.

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Chapter 3: Increased Phosphorylation (p) and Function of pAKT in Rat Urinary Bladder with Cyclophosphamide (CYP)-Induced Cystitis

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Abstract

AKT phosphorylation following peripheral nerve injury or inflammation may play a role in somatic pain processes and visceral inflammation. To examine such a role in bladder inflammation and function we induced bladder inflammation in adult female Wistar rats (200-300g) by injecting CYP intraperitoneally at acute (150 mg/kg; 4hr), intermediate (150 mg/kg; 48 hr) and chronic (75 mg/kg; every third day for 10 days) time points. Western blot analyses of whole urinary bladders showed significant increases ($P \leq 0.01$) of pAKT at all time points; however, the magnitude of pAKT expression varied with duration of CYP-treatment. Immunohistochemical analyses of pAKT-immunoreactivity (IR) cryostat bladder sections demonstrated duration-dependent, significant ($p \leq 0.01$) increases in pAKT - IR in both the urothelium and detrusor smooth muscle of CYP-inflamed bladders. Additionally, a suburothelial population of pAKT-IR positive macrophages (CD68-positive) was present in chronic CYP-treated bladders. The functional role of pAKT in micturition was evaluated using open, conscious cystometry with continuous instillation of saline in conjunction with administration two different inhibitors of AKT phosphorylation (AKT inhibitor IV, 0.6 μ g/10 μ l and Deguelin, 1.0 μ g/ 10 μ l) or vehicle (1% DMSO in saline) in control (no inflammation) and CYP (48 hr)-treated rats. Bladder capacity, void volume and intercontraction void interval increased significantly ($P \leq 0.05$) following intravesical instillation of AKT Inhibitor IV or Deguelin in CYP (48 h)- treated rats or following instillation of AKT Inhibitor IV in control rats. These results suggest pAKT expression in the urinary bladder

contributes to the functional micturition changes exhibited with urinary bladder inflammation.

Introduction

AKT is a putative cellular survival signal (14). Recent studies have demonstrated novel roles for activated (phosphorylated; p) AKT in the initiation and maintenance of neuropathic pain and visceral inflammation (36, 42, 43, 52). Immunohistochemical techniques show AKT activation in pain transducing c-fiber primary afferents and dorsal horn neurons following peripheral neuronal injury or tissue inflammation (19, 41, 42, 43, 52). In visceral inflammation, pAKT levels increase in the spinal cord dorsal horn following TNBS-induced colitis (36). Chung et al. (12) previously detected increases of pAKT levels with cyclophosphamide (CYP)-induced bladder inflammation and determined that inflammation-induced elevation of pAKT levels was dependent on the presence of NGF, as was increased levels of pERK1/2 and pJNK.

Expressional and functional studies demonstrate the physiologic roles of NGF and associated receptors (TrkA, TrkB and p75^{NTR}) in micturition reflex pathways with and without inflammation (11, 15, 29, 32, 37, 40, 51, 53, 56). For example, direct application of NGF to the detrusor smooth muscle, lumen of the bladder, lumbosacral intrathecal space or adenovirus-induced NGF expression in the detrusor smooth muscle causes bladder hyperreflexia, sensitization of bladder afferents and increases FOS and calcitonin gene-related peptide (CGRP) expression in the lumbosacral spinal cord in response to bladder distension (11, 15, 29, 53, 56). Transgenic mice overexpressing NGF under the urothelium-specific uroplakin II promoter exhibit hyperinnervation of unmyelinated and myelinated sensory nerves and sympathetic nerves in the suburothelial plexus, as well as increased urinary bladder reflex activity and referred somatic hypersensitivity in the

pelvic region (40). Conversely, agents that decrease NGF in the urinary bladder can reduce or attenuate chemically-induced bladder hyperreflexia (15, 23).

AKT, ERK1/2 and JNK are all potential signaling pathways downstream of NGF/receptor signaling in the inflamed urinary bladder with inflammation (12). Corrow and Vizzard (13) demonstrated a functional role for pERK in micturition reflexes as treatment with an upstream inhibitor of ERK phosphorylation significantly decreased CYP-induced bladder hyperreflexia in rats. This study addresses the contribution of AKT signaling in bladder function.

Interstitial cystitis (IC)/bladder pain syndrome (BPS) is a chronic urinary bladder syndrome of unknown etiology with symptoms of urgency, frequency, nocturia, suprapubic and pelvic pain (35). Bladder biopsies and urine from IC/BPS patients express elevated levels of NGF suggesting that neurotrophin-induced changes within the bladder and/ or micturition reflex pathways may contribute to IC/BPS symptoms (30, 33). These factors combined with the numerous reports implicating AKT signaling in sensory pain processes or visceral inflammation (36, 42, 43, 52), especially in the presence of NGF (12), suggest that AKT signaling may contribute to bladder dysfunction subsequent to NGF receptor activation. To begin addressing the role of AKT signaling in urinary bladder inflammation, we used a CYP-induced model of bladder inflammation and determined: 1) pAKT protein expression and regulation in the urinary bladder following CYP treatment of varying duration using western blot analyses, immunohistochemistry and semiquantitative image analyses and 2) the functional effects of the upstream inhibitors of AKT phosphorylation (AKT Inhibitor IV and Deguelin) on bladder function

in control (non-inflamed) and CYP-treated female rats using open-void continuous cystometry in conscious rats.

Materials and Methods

Animals

Adult female Wistar rats (200-300 g), purchased from Charles River Canada (St. Constant, PQ, Canada), were housed two per cage and maintained in standard laboratory conditions with free access to food and water. The University of Vermont Institutional Animal Care and Use Committee approved all animal use procedures (protocol 08-085). Animal experimentation was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Induction of CYP-induced cystitis

Rats were anesthetized with isoflurane (2%) and received intraperitoneal (i.p.) injection(s) of CYP (Sigma Aldrich, St. Louis, MO) to produce urinary bladder inflammation. To induce chronic bladder inflammation, CYP was injected (75 mg/kg; i.p.) every third day for 10 days with euthanasia occurring on the tenth day (2, 13, 27). To induce acute bladder inflammation, CYP was injected (150 mg/kg; i.p.) with euthanasia occurring 4 or 48 h after injection (2, 13, 27). Control rats received no treatment. For conscious cystometry studies, rats received CYP as described with bladder function testing occurring 48 h after injection.

Western Blotting for pAKT Expression in Whole Urinary Bladder

Whole urinary bladders were homogenized separately in tissue protein extraction agent (T-PER; Roche, Indianapolis, IN), a mild zwitterionic dialyzable detergent in 25 mM bicine, 150 mM sodium chloride (pH 7.6) containing a protease inhibitor mix (Sigma-Aldrich, St. Louis, MO; 16 µg/ml benzamidine, 2 µg/ml leupeptin, 50 µg/ml lima

bean trypsin inhibitor and 2 µg/ml pepstatin A) and aliquots were removed for protein assay. Samples (25 µg) were suspended in sample buffer for fractionation on gels and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and efficiency of transfer was evaluated. Membranes were blocked for 1 h in a solution of 5% milk in Tris-buffered saline with 0.1% Tween for 4 h at room temperature.

Membranes were incubated in anti-pAKT (1:1,000; 736Ell, Cell Signaling Technology, Danvers, MA) in 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween. Washed membranes were incubated in a species-specific secondary antibody (1:2,000; goat anti-rabbit horseradish peroxidase) in 5% milk in Tris-buffered saline with 0.1% Tween for 2 h at room temperature for enhanced chemiluminescence detection (Pierce, Rockford, IL). Blots were exposed to Biomax film (Kodak, Rochester, NY) and developed. The intensity of each band was analyzed, and background intensities were subtracted using Un-Scan It software (Silk Scientific, Orem, UT). Western blot analysis of AKT (1:1,000; Cell Signaling Technology) in samples was used as a loading control.

Immunohistochemical localization of pAKT in cryostat bladder sections

The bladders were rapidly dissected, placed in 4% paraformaldehyde followed by overnight incubation in 30% sucrose in 0.1M phosphate buffered-saline (PBS) for cryoprotection. Tissue was frozen in optimal cutting temperature (O.C.T.) compound, sectioned (20 µm) on a freezing cryostat and mounted directly onto gelled (0.5%) microscope slides (2, 10, 13). Sections were incubated overnight at room temperature in rabbit anti-pAKT (1:500; D9, Cell Signaling Technology) diluted in 1% goat serum and 0.1 M phosphate buffer. After overnight incubation, sections were washed (3 x 10 min)

with 0.1 M PBS (pH 7.4). Sections were then incubated with goat anti- rabbit Cy3-conjugated secondary antibody (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at room temperature followed by washes (3 x 10 min) with PBS and coverslipping with antifade medium (Citifluor, Fisher Scientific, Pittsburgh, PA). Control tissue sections were incubated with 1% goat serum and 0.1 M phosphate buffer alone (no primary antibody), followed by normal washing and incubation with secondary antibodies in order to evaluate background staining levels. In the absence of primary antibody, no positive immunostaining was observed.

To determine the cellular identity of pAKT-immunoreactive inflammatory cell infiltrates, some bladder sections were also immunostained with CD68 (ED1 rat homologue; 1:750 AbD Serotec, Raleigh, NC), a marker of macrophages. Urinary bladder sections were incubated in a cocktail of primary antibodies against pAKT and CD68 followed by incubation in species-specific secondary antibodies (Cy2 for CD68; 1:200; Jackson ImmunoResearch Laboratories) and processed as described above.

Visualization and semi-quantitative analysis of pAKT in urothelium and detrusor smooth muscle

pAKT-immunoreactivity (IR) in bladder sections was visualized and images were captured using an Olympus fluorescence photomicroscope (Optical Analysis Corporation, Nashua, NH). The filter was set to an excitation range of 560-569 nm and an emission range of 610-655 nm for visualization of Cy3. Images were captured, acquired in tagged image file format (TIFF) and imported into image analysis software (Meta Morph, version 4.5r4; University Imaging, Downingtown, PA) (2, 10, 13). The

free hand drawing tool was used to select the urothelium and the urothelium was measured in total pixels area (2, 10, 13). To evaluate staining in the detrusor smooth muscle, being careful to avoid non-detrusor structures (e.g., blood vessels, inflammatory cells), a rectangle of fixed dimension (125 x 125 pixels) was placed on the section according to random x and y coordinates. This process was repeated 5 times for each image without overlap (10, 26). A threshold encompassing an intensity range of 100-250 grayscale values was applied to the region of interest in the least brightly stained condition first. The threshold was adjusted for each experimental series using concomitantly processed negative controls as a guide for setting background fluorescence. The same threshold was subsequently used for all images.

Immunoreactivity was considered to be positive only when the staining for pAKT exceeded the established threshold. Percent pAKT-IR above threshold in the total area selected was calculated. For the detrusor smooth muscle, the percent pAKT-IR above threshold was calculated by averaging the data collected from all rectangles placed on the image (10, 26).

Assessment of immunohistochemical staining in urinary bladder regions

Immunohistochemistry and subsequent evaluation of pAKT-IR in bladder sections or whole mount preparations were performed on control and experimental tissues simultaneously to reduce the incidence of staining variation that can occur between tissues processed on different days. Staining in experimental tissue was compared with that in experiment-matched negative controls. Urinary bladder sections or

whole mounts exhibiting immunoreactivity that was greater than background level in experiment-matched negative controls were considered positively stained.

Immunohistochemical localization of pAKT in suburothelial nerve plexus in urinary bladder whole mounts

The urinary bladder was dissected rapidly and placed into oxygenated (95% O₂ and 5% CO₂) physiologic saline solution (119.0 mmol NaCl, 4.7 mmol KCl, 24.0 mmol NaHCO₃, 1.2 mmol KH₂PO₄, 1.2 mmol MgSO₄*7H₂O, 11.0 mmol Glucose) (10, 27, 54). Starting at the urethra, a midline incision was made through the bladder. It was then pinned flat onto a silicon-coated plate (Sylgard®, Dow Corning Corporation, Midland, MI), maximally stretched, and then fixed in 2% paraformaldehyde + 0.2% picric acid for 1.5 h. After fixation, the urothelium was separated from the detrusor layer using fine-tip forceps, iris scissors and a dissecting microscope as previously described (10, 27, 54). Notches were made in the region of the bladder neck in order to track orientation and assess regional immunoreactivity of the bladder. Urothelium and bladder musculature were processed for pAKT-IR as described previously. In some whole mounts processed for pAKT-IR, nerve fibers in the suburothelial nerve plexus were also stained with the pan-neuronal marker protein gene product (PGP9.5, 1: 3000; AbD Serotec, Raleigh, NC) to determine potential expression of pAKT in suburothelial nerve fibers and visualized with Cy2-conjugated species-specific secondary antibodies (1:200; Jackson ImmunoResearch Laboratories).

Visualization of pAKT-IR in suburothelial plexus in bladder whole mounts

Whole mount tissues from control and experimental groups were examined using an Olympus fluorescence photomicroscope (Optical Analysis Corporation) with a multiband filter set for simultaneous visualization of the Cy3 and Cy2 fluorophores. Cy2 was viewed using a filter with an excitation range of 447-501 nm and an emission range from 510-540 nm.

Intravesical Catheter Implant

A lower midline abdominal incision was performed under general anesthesia with 2-3% isoflurane using aseptic techniques (2, 9, 23, 26). The end of polyethylene tubing (PE-50, Clay Adams, Parsippany, NJ) was flared with heat, inserted into the dome of the bladder and secured in place with a 6-0 nylon purse-string suture (2, 9, 23, 26). The distal end of the tubing was sealed and tunneled subcutaneously to the back of the neck where it was externalized, out of the animal's reach (2, 9, 23, 26). Rats received buprenorphine (0.05 mg/kg, s.c.) starting at the time of surgery and then every 8-12 h postoperatively for a total of 4 doses. Animals were maintained for 96 h after surgery before conscious cystometry was initiated to ensure complete recovery and absence of post-operative analgesics.

Conscious cystometry with continuous intravesical infusion of saline and inhibition of AKT phosphorylation

The effects of pAKT on bladder function in control (no inflammation) and CYP-treated rats (48 h) were evaluated with upstream inhibitors of AKT phosphorylation, AKT Inhibitor IV (0.6 µg/ 10 µl; EMD4Biosciences, EMD Chemicals, Gibbstown, NJ) and Deguelin (1.0 µg/ 10 µl; EMD4Biosciences, EMD Chemicals) using conscious

cystometry and continuous infusion of intravesical saline. During cystometry, unrestrained and conscious rats were placed in a recording cage over a scale and pan in order to collect and measure voided urine. To elicit repetitive bladder contractions, room temperature saline was infused at a constant rate (10 ml/h). At least six reproducible micturition cycles were recorded after an initial stabilization period (15 - 30 min). Intravesical pressure changes were recorded using a Small Animal Cystometry System (Med Associates, Inc., St. Albans, VT) (2, 9, 13, 26). Filling pressure, pressure threshold for voiding, maximal voiding pressure and intercontraction interval were evaluated. Non-voiding bladder contractions (NVCs), defined as rhythmic intravesical pressure increases 7 cm H₂O above baseline without the release of fluid from the urethra, were also determined per voiding cycle. Bladder capacity was measured as the amount of saline infused into the bladder at the time when micturition commenced (7, 22).

To evaluate the effects of pAKT on bladder function, rats were anesthetized (1-2% isoflurane) and vehicle (1% dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) in saline) or specific upstream inhibitors of AKT phosphorylation, AKT Inhibitor IV (EMD4Biosciences, EMD Chemicals; 0.6 µg/ 10 µl) or Deguelin (EMD4Biosciences, EMD Chemicals; 1.0 µg/ 10 µl), was intravesically infused for 30 min. The concentrations of inhibitors selected for evaluation were based upon previous studies (42, 43, 52). Prior to intravesical drug infusion, the bladder was manually emptied using the Credé maneuver. Bladders were then infused with approximately 1 ml (less than bladder capacity to not elicit a bladder contraction and expulsion of instillate) of vehicle (1% dimethylsulfoxide (DMSO; Sigma-Aldrich) in saline), AKT Inhibitor IV

(EMD4Biosciences, EMD Chemicals; 0.6 μ g/ 10 μ l) or Deguelin (EMD4Biosciences, EMD Chemicals; 1.0 μ g/ 10 μ l) according to prior published studies (2, 9, 26). Rats remained anesthetized (1-2% isoflurane) to subdue the micturition reflex and prevent expulsion of drug from the bladder. To avoid potential variation resulting from circadian rhythms, experiments were conducted at similar times of the day (16). At the conclusion of the study, rats were euthanized as described above. Baseline recordings and effects of AKT inhibitor IV and Deguelin were evaluated in the same rats.

Exclusion Criteria

Rats were removed from the study when adverse events occurred that included: \geq 20% reduction in body weight post surgery, a significant postoperative event, lethargy, pain, or distress not relieved by our IACUC-approved regimen of postoperative analgesics or hematuria in control rodents (2, 15, 26). In the present study, no rats were excluded from the study or from analysis due to any of these exclusion criteria. In addition, behavioral movements such as grooming, standing, walking, and defecation rendered bladder pressure recordings during these events unusable.

Materials

AKT Inhibitor IV and Deguelin were prepared as concentrated stock solutions in DMSO, aliquoted and stored at -20°C until usage. Aliquots were diluted with saline to achieve final concentration.

Figure Preparation

Digital images were obtained using a charge-coupled device camera (MagnaFire SP, Optonics; Optical Analysis Corporation) and LG-3 frame grabber attached to an

Olympus fluorescence photomicroscope (Optical Analysis Corporation). Exposure times were held constant when acquiring images from control and experimental animals processed and analyzed on the same day. Images were imported into a graphics editing program (Adobe Photoshop, version 8.0, Adobe Systems, San Jose, CA) where groups of images were assembled and labeled.

Statistical Analyses

All values represent mean \pm S.E.M. Data were compared with analysis of variance (ANOVA) or repeated measures ANOVA, where appropriate. When F ratios exceeded the critical value ($P \leq 0.05$), the Newman-Keul's or Dunnett's post hoc tests were used to compare group means.

Results

pAKT protein expression and effects of cyclophosphamide (CYP)-induced cystitis in the whole rat urinary bladder

pAKT protein expression increases significantly ($P \leq 0.01$) following 4 h (5.7 - fold), 48 h (3.4-fold) and chronic (2.9-fold) CYP-treatment as determined with western blot analyses (Fig. 1). 4 h CYP-treatment induced the greatest upregulation of pAKT expression which was significantly ($P \leq 0.01$) greater than expression after 48 h (1.7 - fold) and chronic (2.0-fold) CYP-treatment (Fig. 1).

pAKT-immunoreactivity (IR) in the urothelium and detrusor after CYP-induced cystitis

Urothelium. Patchy, low intensity basal pAKT-IR was present in the urothelium of control rat bladders (Fig. 2, A and B). CYP-treatment increased urothelial pAKT-IR significantly ($P \leq 0.01$) at the 48 h (2.3-fold) time point, but not 4 h or chronic time points examined (Figs. 2 and 4A). pAKT-IR at the 48 h time point was also significantly ($P \leq 0.01$) greater than 4 h (1.5-fold) and chronic (3.6-fold) CYP-treatment (Figs. 2, C-H and 4A). Although 4 h CYP-treatment did not significantly increase pAKT-IR versus control, pAKT expression did increase significantly ($P \leq 0.01$) in comparison to chronic (2.3-fold) CYP-treatment (Figs. 2, C,D,G,H and 4A). Double-labeling experiments demonstrated that the pAKT-immunoreactive suburothelial inflammatory cells present in chronic CYP-treated bladders (Fig. 2H) also stained positively for CD68 (ED1 rat homologue), a marker of macrophages (data not shown).

Detrusor. The expression of pAKT-IR was very low to absent in the detrusor smooth muscle of control rat bladders (Fig. 3A). Forty-eight (48) h CYP-treatment significantly

($P \leq 0.05$; 2.5-fold) increased detrusor pAKT-IR (Figs. 3C and 4B). There was no difference between control, 4 h or chronic pAKT-IR expression in the detrusor (Figs. 3A, B, D and 4B).

Bladder whole mounts. pAKT-IR was not observed in the suburothelial nerve plexus in control or CYP-treated whole mount bladder preparations. The suburothelial nerve plexus did exhibit PGP9.5-IR; however, colocalization with pAKT-IR was not observed with indirect immunofluorescence techniques (data not shown). Whole mount preparations exhibited urothelial and detrusor smooth muscle pAKT staining consistent with staining observed in cryostat bladder sections described above (data not shown).

Effect of upstream inhibition of AKT phosphorylation on bladder function in rats with and without CYP-induced cystitis

Conscious, open outlet cystometry with continuous intravesical infusion of saline was performed in control and CYP-treated (48 h) rats to establish baseline voiding frequency, bladder capacity and void volume. After baseline bladder function was established, AKT Inhibitor IV (0.6 $\mu\text{g}/10\text{ }\mu\text{l}$), Deguelin (1.0 $\mu\text{g}/10\text{ }\mu\text{l}$) or 1% DMSO in saline (vehicle) was intravesically instilled for 30 minutes under 2% isoflurane. The same rats subsequently underwent conscious cystometry for a second time to establish the effects of inhibiting AKT phosphorylation using AKT Inhibitor IV or Deguelin or vehicle on normal bladder function or CYP-induced bladder hyperreflexia. The 48 h time point of CYP-induced cystitis was evaluated using inhibitors of AKT phosphorylation because western blot and immunohistochemical techniques both demonstrated significant increases in pAKT expression in the urinary bladder with this duration of CYP treatment.

Control (no inflammation). There was no difference between baseline recordings of control rats pre AKT inhibitor IV (no treatment), pre Deguelin (no treatment) or pre vehicle treatments; therefore, these three groups were combined into one group referred to as the pre treatment group. Treatment with AKT Inhibitor IV, but not Deguelin, significantly ($P \leq 0.01$) increased intercontraction interval (1.4-fold), bladder capacity (1.4-fold) and void volume (1.3-fold) in comparison to baseline recordings (no treatment) in the same rats (Fig. 5 A-C). There were no changes in filling, threshold or peak micturition pressures following intravesical instillation of AKT Inhibitor IV, Deguelin or vehicle (Table 1). Residual volume in control rats before or after AKT Inhibitor IV or Deguelin treatment was minimal ($\leq 10 \mu\text{l}$).

CYP treatment. As previously demonstrated (2, 9, 23, 26), and confirmed here, CYP treatment (48 h) increased void frequency and decreased bladder capacity, intercontraction interval, and void volume (Figs. 6A and 7A). Additionally, CYP treatment increased filling, threshold, and micturition pressures (Table 1). There was no difference between baseline recordings of CYP-treated rats prior to AKT inhibitor IV, Deguelin or vehicle treatments; therefore, these three groups were combined into one group referred to as the pre treatment group. Treatment with AKT Inhibitor IV (Figs. 6 and 8) or Deguelin (Figs. 7 and 8) significantly ($P \leq 0.05$) decreased void frequency as measured by increased intercontraction interval (1.9-fold, AKT Inhibitor IV; 2.0-fold, Deguelin), increased bladder capacity (1.9-fold, AKT Inhibitor IV; 2.0-fold, Deguelin) and increased void volume (2.0-fold, AKT Inhibitor IV; 2.2-fold, Deguelin) in comparison to baseline recordings (no treatment) for each treatment. Effects of AKT

Inhibitor IV or Deguelin on bladder function in CYP-treated or normal (see above) rats persisted for at least for 2 h and recovery to baseline recordings (no treatment) was not observed. Residual volume in CYP (48 h) - treated rats before or after AKT Inhibitor IV or Deguelin treatment was minimal ($\leq 10 \mu\text{l}$).

Discussion

These studies demonstrate basal activation (phosphorylation) of AKT in the urinary bladder, and inflammation-enhanced phosphorylation of AKT with CYP-induced cystitis. To our knowledge, these studies are the first to demonstrate a functional role for AKT signaling in female, rat micturition pathways with normal or inflamed urinary bladders. Studies have previously demonstrated changes of AKT activation levels in primary sensory neurons, dorsal horn neurons and urinary bladder (12, 36, 42, 43) following tissue inflammation. The present studies complement previous findings and demonstrate a functional significance for AKT signaling in bladder reflex pathways. pAKT may be a functional mediator of inflammation-induced bladder dysfunction because pAKT: 1) is expressed at low levels in the urothelium of control urinary bladders; 2) increases in the urinary bladder after CYP treatment of varying duration; 3) increases in the urothelium and detrusor with acute CYP treatment; 4) is present in suburothelial inflammatory cells with chronic CYP-treatment and, most interestingly; 5) inhibition of AKT phosphorylation with two different inhibitors of AKT phosphorylation, AKT Inhibitor IV and Deguelin, increases (2.0-fold) bladder capacity in rats with urinary bladder inflammation induced by cyclophosphamide treatment. Therefore, the AKT signaling pathway may represent a novel target for pharmaceutical intervention and improving bladder function with respect to bladder inflammation.

pAKT is classically known as a putative cellular survival signal (14). More recently, studies have demonstrated novel roles for AKT signaling in altered sensory/pain processing. For example, basal AKT phosphorylation is present in rodent

lumbosacral dorsal root ganglia (DRG) (41, 42) and superficial laminae of the spinal cord (19, 43) and significantly increases following peripheral nerve injury or inflammation (19, 41, 42, 43, 52). Additionally, colocalization of pAKT with markers of unmyelinated pain transducing c-fibers such as IB4, TrkA, CGRP and transient potential vanilloid-1 channel (TRPV1) (41, 42, 52, 55) has been demonstrated. Specific inhibitors of AKT signaling including AKT Inhibitor IV, Deguelin, SH-6 or NL-71-101 can attenuate mechanical hyperalgesia resulting from intradermal hind paw capsaicin injection (42, 43) or sciatic nerve ligation (52) suggesting a nociceptive role for AKT signaling in peripheral inflammation or injury.

In addition to mediation of somatic pain processes, PI3-K/ AKT signaling may also mediate visceral inflammation. For example, basal pAKT expression in the superficial dorsal horn of lumbosacral spinal cord increases significantly following TNBS-induced colitis (36). Therefore AKT-mediated inflammation in other pelvic viscera such as the bladder seems plausible and pAKT has been localized previously to the mouse urothelium (48).

The present studies demonstrate a functional role for pAKT in bladder inflammation because pAKT expression not only increases with CYP- treatment in the urinary bladder, but also two different upstream inhibitors of AKT phosphorylation, AKT Inhibitor IV and Deguelin, independently decreased voiding frequency, and increased bladder capacity and void volume following CYP-induced bladder inflammation. These data suggest that PI3-K/ AKT signaling may mediate or contribute to functional aspects of IC/BPS, a chronic bladder syndrome with symptoms of urgency, frequency, nocturia,

suprapubic and pelvic pain (35). Although the etiology and pathogenesis of IC/BPS are unknown, numerous theories, including infection, autoimmune disorder, toxic urinary components, deficiency in bladder wall lining, and neurogenic causes have been proposed (17, 24, 25, 34, 35, 39). We hypothesized that pain associated with IC/BPS involves alteration of visceral sensation/bladder sensory physiology. Altered visceral sensations from the bladder (i.e. pain at low or moderate bladder filling) that accompany IC/BPS may be mediated by many factors including changes in the properties of peripheral bladder afferent pathways such that bladder afferent neurons respond in an exaggerated manner to normally innocuous stimuli (allodynia). These changes may be mediated, in part, by inflammatory changes in the urinary bladder. Potential mediators of bladder inflammation are numerous: cytokines (18, 28, 31), neuropeptides (8, 50), neuroactive compounds (6), chemokines (2, 38, 49, 54) and growth factors (51, 53, 56) and signaling molecules such as pERK1/2 (13) and pAKT (present study; 12).

Considering urothelial levels of pAKT in inflamed and non-inflamed bladders, AKT signaling may contribute to functional bladder sensory physiology via urothelium-mediated mechanisms. The urothelium, once thought to provide an impermeable barrier only, is now suggested to have “neuron-like” properties such as plasticity and sensory, transductive and signaling capabilities, especially in the context of bladder inflammation (1, 3, 4, 5, 44, 45, 46, 47). Urothelial cells share a number of similarities with sensory neurons, including the expression of receptors such as purinergic, norepinephrine, acetylcholine, neuropeptide- and protease-activated receptors, acid sensing ion channels, neurotrophin receptors and transient receptor potential (TRP) channels (1, 3, 4, 5, 44, 45,

46, 47). Because of functional receptor expression and secretion capabilities, urothelial-mediated communication with the detrusor smooth muscle, suburothelial plexus and/ or interstitial cells has been suggested (1, 4). Therefore, inflammatory signaling cascades may activate AKT signaling and thus facilitate the release of urothelial derived mediators such as ATP, NO or chemokines that may then influence the suburothelial nerve plexus to affect micturition function via urothelium-to-neuron- communication (1, 2, 3, 4, 5). Alone, or in combination with urothelium-mediated mechanisms, pAKT expression in the detrusor smooth muscle may contribute to CYP-induced bladder hyperreflexia. Alternative roles for the phosphorylation of AKT in detrusor smooth muscle following cystitis may involve NGF-induced increases in type I collagen and resultant bladder hypertrophy via activation of AKT (12). Future studies may address NGF-induced release of ATP or NO via AKT signaling using cultured rat urothelial cells and pharmacological blockade of AKT phosphorylation.

Potential upstream activators of AKT phosphorylation during inflammation include, but are not limited to, growth factors, cytokines and reactive oxygen species. NGF is a bladder inflammatory mediator of particular interest because NGF protein levels are increased in the urine and urothelium of patients with IC/BPS (30, 33), and NGF is an important mediator of both somatic and visceral inflammation. NGF interactions with the high affinity receptor, TrkA, are known to activate the PI3-K/ AKT ERK1/2 and JNK signaling pathways. Chung et al. (12) demonstrate that AKT and ERK1/2 phosphorylation in the urinary bladder with CYP-induced bladder inflammation is dependent on the presence of NGF suggesting that NGF constitutes a major upstream

activator of AKT phosphorylation in bladder inflammation. Numerous rodent studies demonstrate the physiologic relevance of NGF signaling in the urinary bladder (11, 15, 23, 29, 40, 53, 56). Previous (13) and present studies now demonstrate that blockade of potential downstream NGF-signaling targets, ERK1/2 or AKT activation, reduces urinary bladder hyperreflexia following CYP-induced cystitis. Whether cross-talk exists between these two pathways in the context of urinary bladder inflammation (12) or whether further improvement in bladder function can be achieved by blocking both AKT and ERK1/2 activation remains to be determined.

In the absence of CYP-induced bladder inflammation, intravesical instillation of AKT Inhibitor IV significantly increased (1.4-fold) bladder capacity in control rats; however, Deguelin treatment was without effect. AKT signaling may not play a major role in non-inflamed bladder function. However, in this study only a single concentration of Deguelin was evaluated so potential effects with higher concentrations are not known. The concentrations of AKT phosphorylation inhibitors used in this study were the same as those used to reduce pain behavior after peripheral nerve injury or inflammation where drugs were administered intrathecally (43, 52) or intradermally (42). Or perhaps AKT Inhibitor IV has greater cell permeability enabling the compound to reach deeper layers of the urothelium, detrusor smooth muscle or suburothelial plexus. Although the exact mechanism of drug action is not known, AKT Inhibitor IV is thought to attenuate not only future phosphorylation of AKT, but is also thought to disrupt phosphorylated forms of AKT activated before and during the time of drug treatment (EMD4Biosciences website). Additionally, Sun et al. (42) and Xu et al. (52) suggest that PI3-K/ AKT

signaling does not influence basal thermal or mechanical sensitivity prior to peripheral inflammatory or mechanical insult. This is consistent with the present studies where more dramatic effects on bladder function were observed in CYP-treated rats following intravesical delivery of inhibitors of AKT phosphorylation.

In the present studies, inhibitors of AKT phosphorylation when used after development of urinary bladder inflammation with CYP-treatment were effective in improving urinary bladder function (i.e., reduce bladder hyperreflexia). Several studies have also demonstrated that prophylactic treatment with AKT Inhibitor IV prior to peripheral injury or inflammation can reduce the subsequent hyperalgesia suggesting a role in the initiation of inflammatory pain (43, 52). To assess the functional contribution of PI3K/ AKT signaling in the initiation of CYP-induced bladder hyperreflexia, in pilot studies, we intravesically infused AKT Inhibitor IV before intraperitoneal CYP injection and development of urinary bladder inflammation. The effectiveness of AKT Inhibitor IV to reduce CYP-induced bladder hyperreflexia (48 h) was highly variable and did not reach statistical significance (data not shown). Possible explanations for differences in the effectiveness of AKT Inhibitor IV to reduce pain behavior or bladder hyperreflexia prophylactically may include different administration routes (e.g. intrathecal or intraplantar vs. intravesical), somatic vs. visceral systems, injury/ inflammation model and/or dosing schedule, or differences between the onset of functional testing after injury or inflammation. Sun et al. (43) and Xu et al. (52) also demonstrate that treatment with AKT Inhibitor IV after peripheral nerve injury or inflammation can reduce hyperalgesia. Our results are consistent with a role for pAKT with pre-existing urinary bladder

inflammation and hyperreflexia further demonstrating the functional relevance of blocking AKT activation (i.e., phosphorylation). It remains to be determined whether inhibition of AKT phosphorylation following CYP-induced cystitis can also reduce referred somatic (i.e., pelvic) pain, which has also been demonstrated to depend upon NGF/Trk interactions (20, 21). Effects of blocking AKT activation on pelvic sensitivity can be evaluated in the future using the NGF-OE mouse model that in addition to demonstrating urinary bladder hyperreflexia also exhibits increased pelvic sensitivity (40).

Conclusions

These studies demonstrate basal expression and increased activation of AKT in the urinary bladder after CYP-induced cystitis. Importantly, a functional role for the phosphorylation of AKT in bladder function in normal rats and those with urinary bladder inflammation induced by CYP has also been demonstrated. Blockade of AKT activation may be an effective target for a pharmacological intervention aimed at improving bladder dysfunction resulting from bladder inflammation.

Grants

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Figures

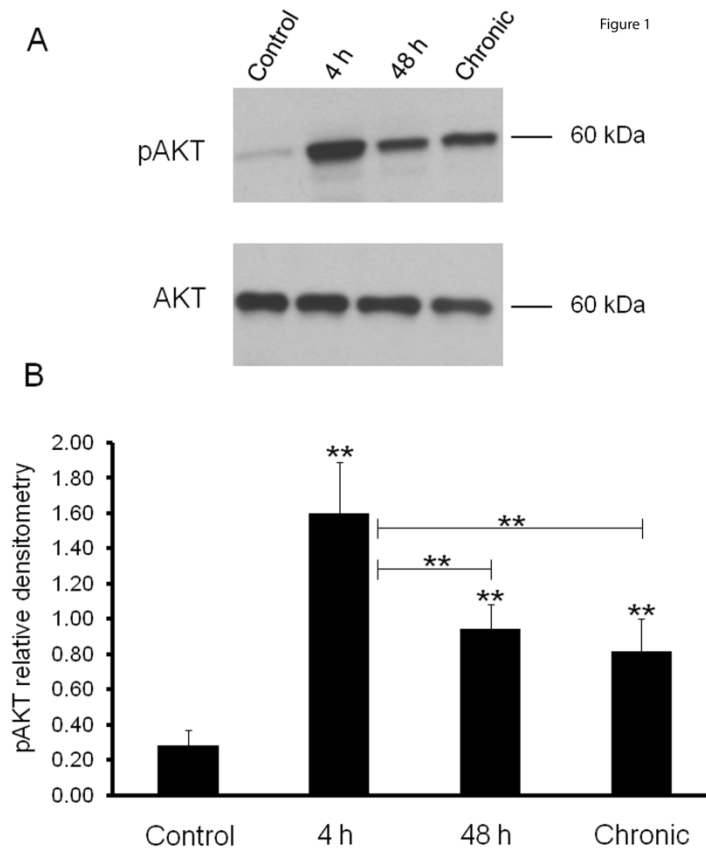


Fig. 1. A: Representative western blot of phosphorylated (p) AKT expression in whole urinary bladders (25 μ g) from control rats and those treated with cyclophosphamide (CYP) for varying duration. Total AKT expression was used as a loading control. B: Summary histogram of relative pAKT band density in each group normalized to total AKT expression in the same samples. 4 h, 48 h and chronic CYP-treatment significantly ($P \leq 0.01$) increased pAKT expression in comparison to control urinary bladder. pAKT expression was significantly increased following 4 h CYP treatment in comparison to 48 h and chronic CYP treatment. (** $P \leq 0.01$, $n = 4$). Values are mean \pm S.E.M.

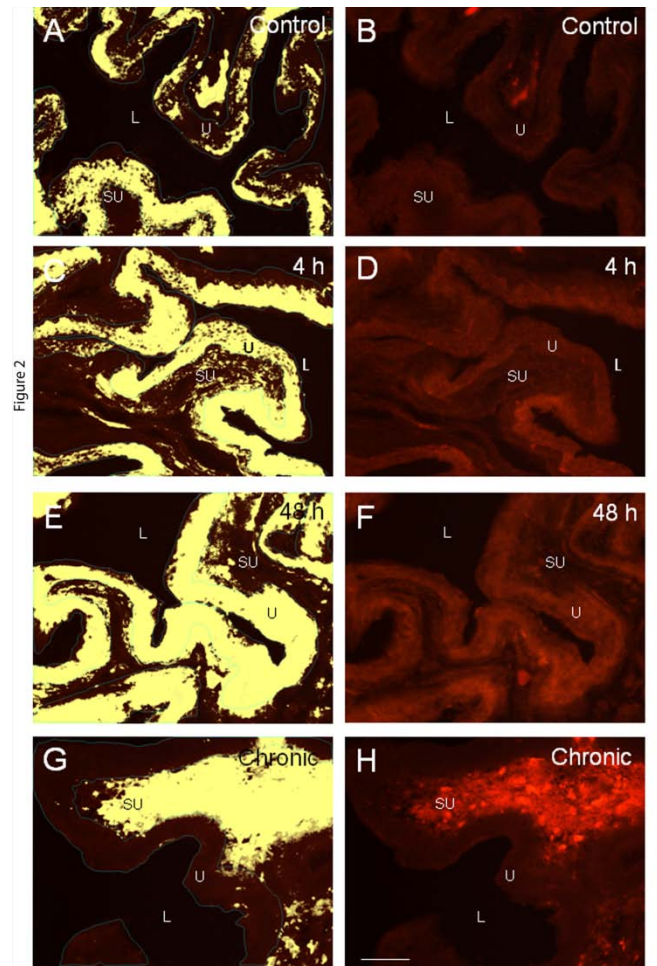


Fig. 2. pAKT expression in the urothelium of control rats and those treated with cyclophosphamide (CYP). A,C,E,G: the urothelium was outlined in green and measured in total pixels per area. To define positive staining, a threshold intensity value was determined using the darkest image and the same threshold was subsequently applied to all images. All pixels above threshold are depicted in yellow. B,D,F,H: Corresponding fluorescence images of pAKT expression in control (B) or after 4 h (D), 48 h (F) and chronic (H) CYP-treatment. U, urothelium; SU, suburothelium; L, lumen. Calibration bar represents 50 μ m.

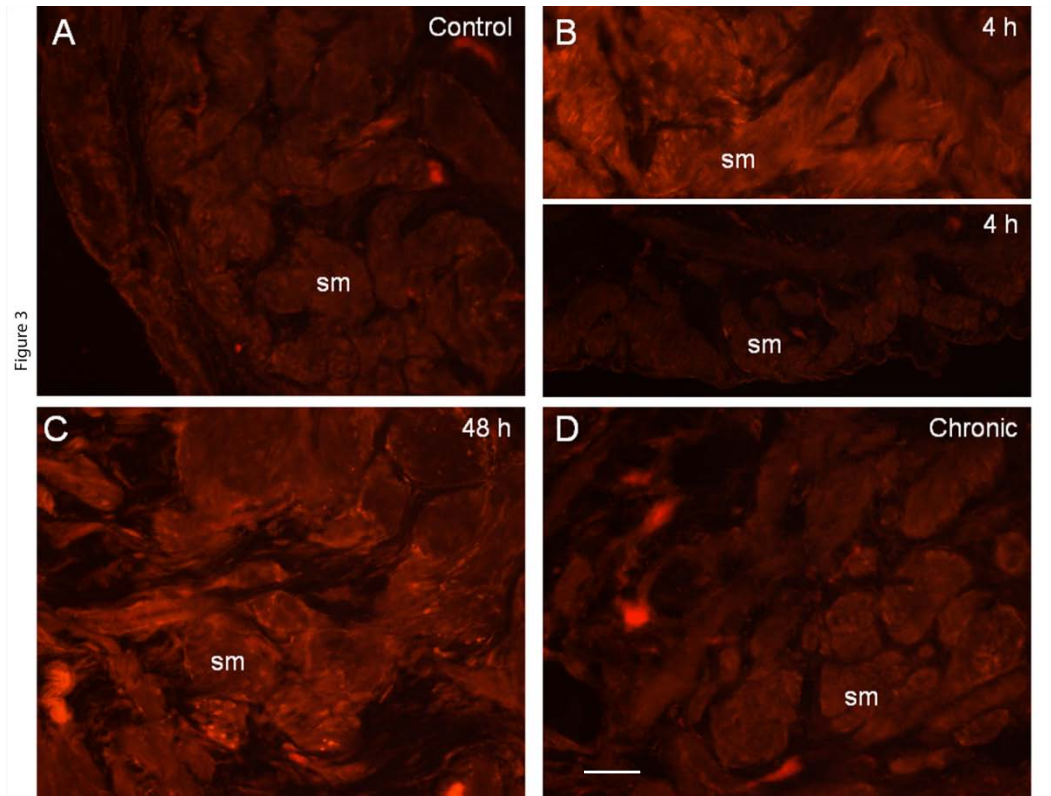


Fig. 3. pAKT expression in the detrusor smooth muscle of control (A) rats and following 4h (B), 48h (C) and chronic (D) treatment. The top and bottom panels in B demonstrate the variability of detrusor smooth muscle staining in 4 h CYP-treated rats. For all images, exposure times were held constant and all tissues were processed simultaneously. sm, detrusor smooth muscle. Calibration bar represents 50 μ m.

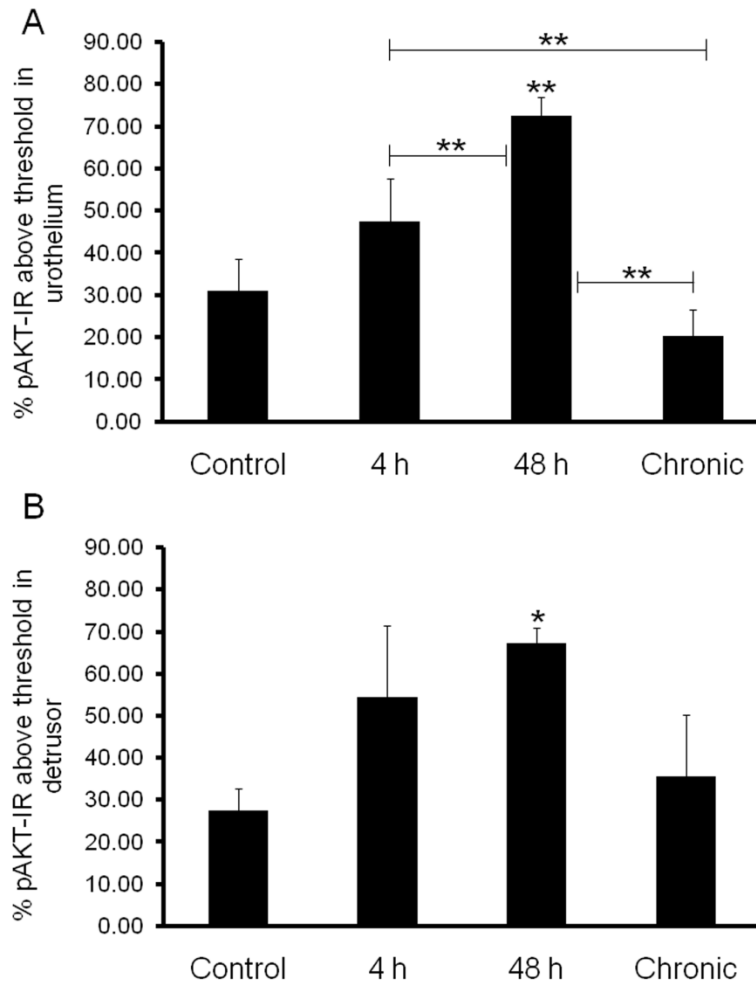


Figure 4

Fig. 4. Summary histogram of pAKT- immunoreactivity (IR) in the urothelium (A) and detrusor (B) of the urinary bladder of control and CYP-treated rats. Percent pAKT-IR above threshold in the urothelium following 48 h CYP treatment was significantly ($**P \leq 0.01$) greater than control, 4 h and chronic CYP-treatment (A). Four (4) h CYP treatment significantly increased percent pAKT-IR in the urothelium compared to chronic CYP-treatment (A). B: Percent pAKT-IR above threshold in the detrusor smooth muscle was significantly ($*P \leq 0.05$) greater following 48 h CYP-treatment. Values are means \pm S.E.M. (n = 4 in each group).

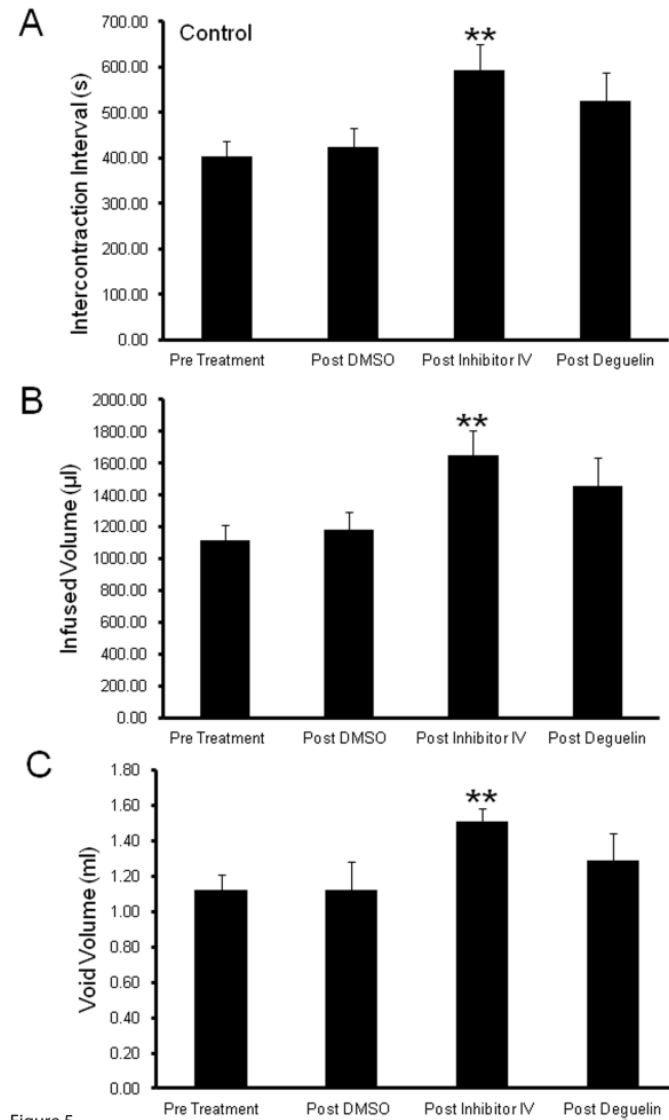


Figure 5

Fig. 5. Summary histograms of the effects of vehicle, AKT Inhibitor IV and Deguelin treatments on intercontraction interval, infused volume and void volume in control (no inflammation) rats. There was no difference between baseline recordings of control rats prior to vehicle, AKT Inhibitor IV or Deguelin treatments; therefore, these three groups were combined into one group: pre treatment. Statistical analyses however, were performed between pre (no treatment) and post treatments within a drug treatment group. Treatment with AKT Inhibitor IV only, significantly ($**P \leq 0.01$) increased intercontraction interval (A), infused volume (B) and void volume (C) in control (no inflammation) rats in comparison to baseline recording prior to AKT Inhibitor IV treatment. Values are means \pm S.E.M.; $n = 4$ in each group.

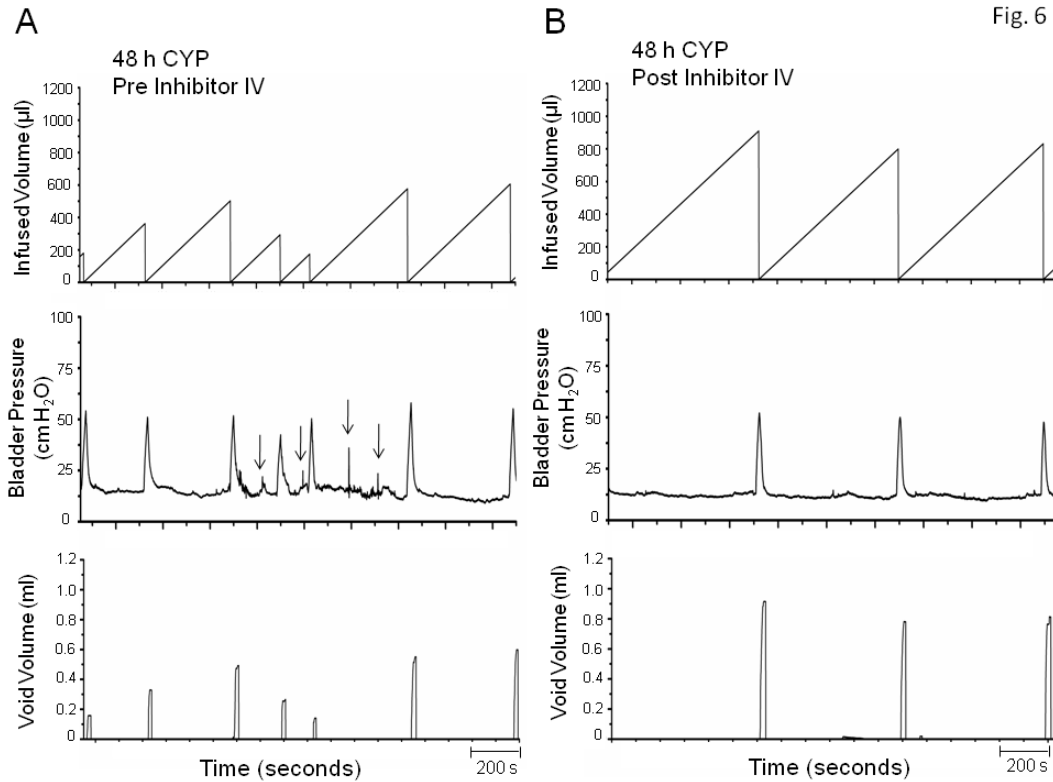


Fig. 6. Representative cystometrogram recordings of effects of blocking upstream AKT phosphorylation with AKT Inhibitor IV in CYP-treated (48 h) rats using continuous intravesical infusion of saline in conscious rats with an open outlet. A, B: Pre AKT Inhibitor IV (no treatment) (A) and post AKT Inhibitor IV treatment (B) in 48h CYP-treated rats with continuous intravesical infusion of saline in conscious rats. Bladder function recordings in A and B are from the same rat. Arrows (A, bladder pressure trace) indicate examples of non-voiding contractions as defined in the methods section.

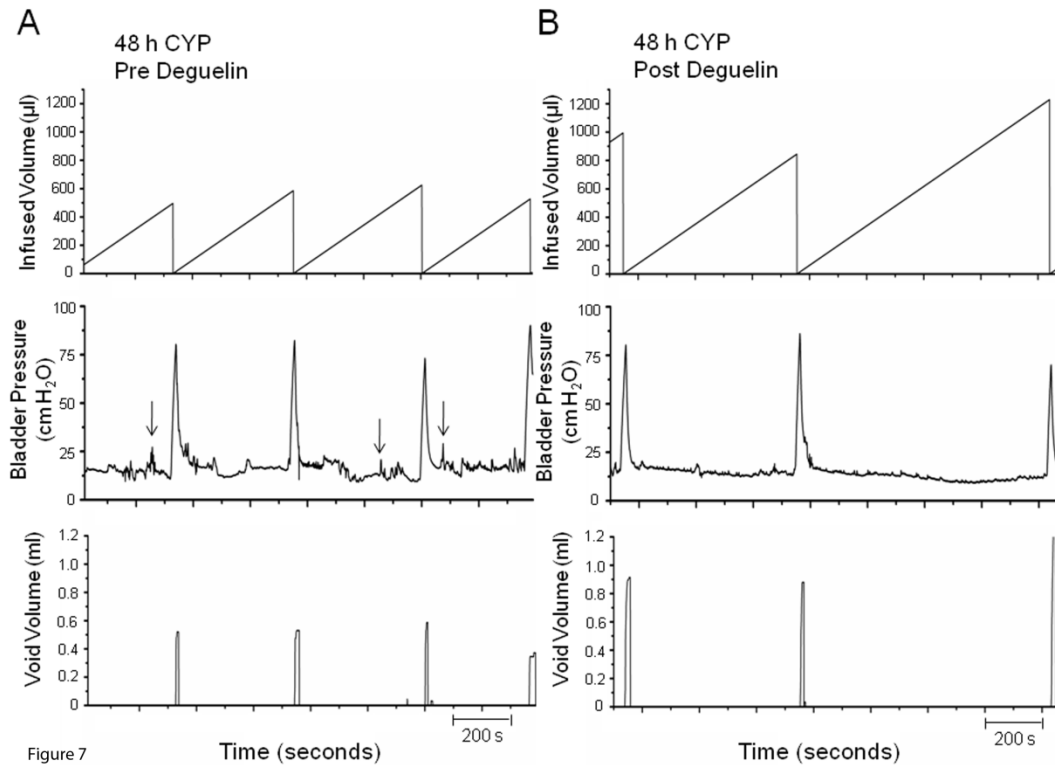


Fig. 7. Representative cystometrogram recordings of effects of blocking upstream AKT phosphorylation with Deguelin in CYP-treated (48 h) rats using continuous intravesical infusion of saline in conscious rats with an open outlet. A, B: Pre Deguelin (no treatment) (A) and post Deguelin treatment (B) in 48h CYP-treated rats with continuous intravesical infusion of saline. Bladder function recordings in A and B are from the same rat. Arrows (A, bladder pressure trace) indicate non-voiding contractions as defined in the methods section.

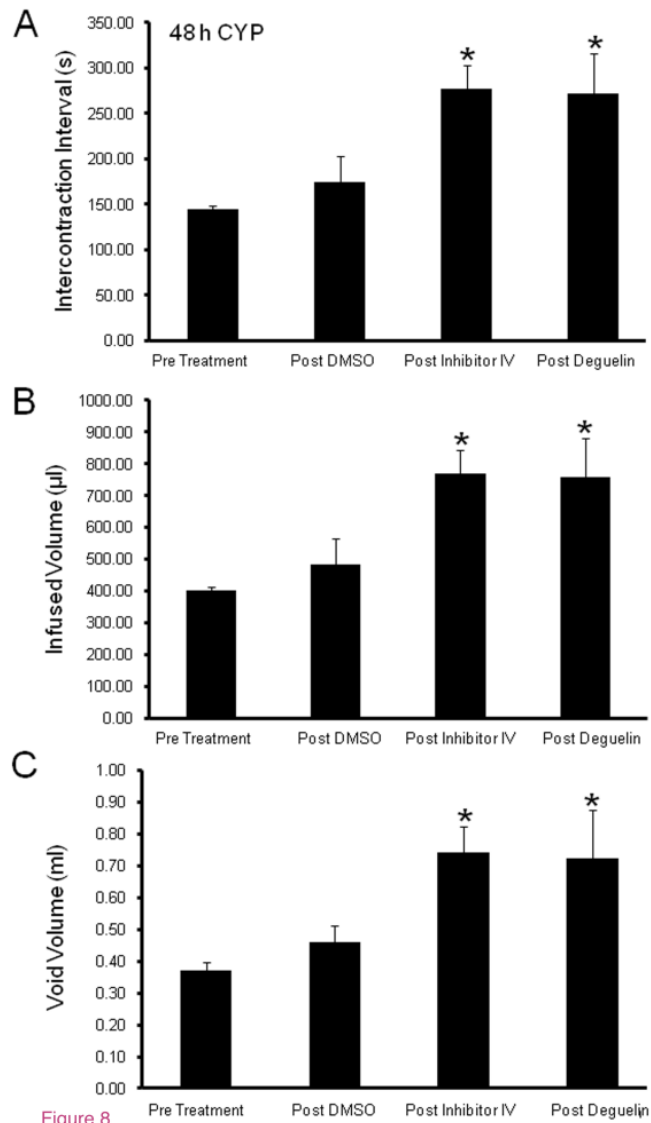


Fig. 8. Summary histograms of the effects of vehicle, AKT Inhibitor IV and Deguelin treatments on intercontraction interval, infused volume and void volume in CYP-treated (48 h) rats. There was no difference between baseline recordings of 48 h CYP-treated rats prior to vehicle, AKT Inhibitor IV or Deguelin treatments; therefore, these three groups were combined into one group: pre treatment. Statistical comparisons however, were performed between pre and post treatments within a drug treatment group (e.g., pre vehicle was compared to post vehicle treatment only). Treatment with AKT Inhibitor IV or Deguelin significantly ($*P \leq 0.05$) increased intercontraction interval (A), infused volume (B) and void volume (C) in CYP-treated (48 h) rats in comparison to pre AKT Inhibitor IV or pre Deguelin treatments. Values are means \pm S.E.M. ($n = 4$ in each group).

Table 1: Mean pressures during cystometry

	Filling Pressure (cm H ₂ O)	Threshold Pressure (cm H ₂ O)	Peak Micturition Pressure (cm H ₂ O)
Control			
Pre Treatment	10.5 ± 0.7	11.8 ± 0.4	43.9 ± 0.6
Post DMSO	12.9 ± 2.2	15.2 ± 1.0	49.1 ± 6.7
Post Inhibitor IV	9.0 ± 0.3	14.2 ± 2.1	40.5 ± 3.8
Post Deguelin	9.0 ± 0.8	11.4 ± 1.4	50.5 ± 5.0
48 h CYP			
Pre Treatment	19.8 ± 2.0	19.8 ± 1.7	60.7 ± 0.7
Post DMSO	22.7 ± 3.4	22.0 ± 4.2	59.1 ± 2.3
Post Inhibitor IV	18.9 ± 3.5	18.5 ± 3.3	62.3 ± 4.6
Post Deguelin	13.0 ± 2.5	16.6 ± 2.7	60.1 ± 5.9

Table 1. Filling, threshold and peak micturition pressures during conscious cystometry for control and 48h CYP-treated rats prior to and after vehicle (1% DMSO in saline), AKT Inhibitor IV or Deguelin treatments. Values are mean ± S.E.M.; n = 4 in each group.

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Chapter 4: Summary and Future Directions

Peripheral inflammation can induce many changes in the bladder and central reflex pathways and is thought to underlie symptoms of various dysfunctional bladder syndromes (e.g., IC/ BPS). These changes are neither fully characterized nor understood. Systemic treatment with cyclophosphamide, an anti- neoplastic agent, produces overt bladder inflammation characterized by infiltration of inflammatory molecules and the presence of hemorrhages on the luminal surface. Functionally, CYP treatment causes intense bladder hyperreflexia and referred somatic pain. Similarities between bladder pathologies and symptoms associated with CYP-induced bladder inflammation suggest that it is an appropriate model to test hypotheses related to inflammation-induced plasticity potentially affecting bladder dysfunction. Although no consensus has been reached regarding the etiology of IC/BPS, examination of the functional roles of inflammatory mediators is valuable because 1) mediators of inflammation such as leukocytes, mast cells, interleukins and NGF are elevated in the bladders and/or urine of patients with IC/BPS (Erickson et al., 1997; Okragly et al., 1999; Lowe et al., 2007), and 2) suppression of inflammatory activation improves bladder function and pain symptoms in rodents with chemically-induced cystitis and, more importantly, in patients with IC/BPS (Ueda et al., 2000; Yoshimura et al., 2002).

Summary and conclusions: Chemokine signaling in the urinary bladder

This dissertation examines the roles of inflammatory mediators belonging to the innate immune system and potential downstream signaling pathways. Specifically, I investigated the role of the chemokine/ receptor pair, CXCL12/CXCR4 and the activated

form of the intracellular signaling molecule, AKT. Chemokines are putative mediators of immune/ inflammatory responses, and they are now receiving considerable attention for potential roles in visceral inflammation and pain modulation. Bladders from control animals (no inflammation) exhibit basal CXCL12 expression in the urothelium, suburothelium and detrusor smooth muscle. The cognate receptor for CXCL12, CXCR4 protein expression is detected in the urothelium only. Consistent with previous demonstrations of inflammation-induced chemokine expression, urinary bladders from CYP-treated animals demonstrate increased CXCL12 and CXCR4 mRNA and protein expression after CYP-induced cystitis. CXCL12 increases throughout the layers of the bladder, but most robustly in the urothelium. Significant CXCL12 increases were detected following 48 h and chronic CYP treatment. CXCR4 mRNA transcript increased following 48h treatment only. Histologically, CXCR4 expression remains limited to the urothelium even after CYP treatment. Neither CXCL12 nor CXCR4 is detected in the suburothelial nerve plexus.

Future Directions: Chemokine signaling in the urinary bladder

We demonstrated the functional role of chemokine signaling in the urinary bladder by combining conscious cystometry with continuous intravesical infusion of saline with CXCR4 receptor blockade. Intravesical infusion of AMD3100, a CXCR4 receptor antagonist, for 30 minutes increases bladder capacity in control rats and reduces CYP-induced bladder hyperreflexia. Considering that CXCR4 expression is limited to the urothelium, and neither CXCL12 nor CXCR4 is detected in the suburothelial plexus,

CXCL12/CXCR4 signaling may influence bladder function via urothelial- mediated mechanisms.

In order to fully assess the contribution of urothelial chemokine signaling in normal or inflamed micturition, additional studies examining chemokine-mediated urothelial release of neuroactive compounds should be performed. Urothelial cells express numerous functional receptors on the cell surface thus enabling the urothelium to respond to various stimuli such as distention or toxic urinary components (Birder, 2006; Apodaca et al., 2007; Birder and de Groat, 2007). Additionally, urothelial cells can release many different signaling molecules such as ATP, NO, neurotrophins, neuropeptides, acetylcholine, prostanoids and cytokines in response various external stimuli (Birder et al., 1998; Birder et al., 2003; Birder, 2006; Apodaca et al., 2007; Birder and de Groat, 2007). Functional receptor expression in conjunction with secretion capabilities allows the urothelium to respond to stimuli and reciprocally communicate with detrusor smooth muscle cells, suburothelial nerve plexus/ or interstitial cells. As such, the urothelium is thought to play a role in normal bladder function, but even more so in the presence of bladder inflammation. Importantly, bladder inflammation can alter urothelial secretion and potentially facilitate bladder dysfunction (Birder, 2006; Apodaca et al., 2007; Birder and de Groat, 2007). A change in the amount or type of substances released by the urothelium may act to sensitize underlying structures (i.e. bladder afferents, interstitial cells or the detrusor smooth muscle). Therefore, it is possible that CXCL12 signaling via CXCR4 expression in urothelial cells may consequently activate downstream targets that promote either the transcription or the release of other

inflammatory mediators or excitatory amino acids. Urothelial derived mediators such as ATP or NO may then influence the suburothelial nerve plexus to affect micturition reflex function.

To assess chemokine-induced secretion of urothelial mediators, the next group of experiments should explore the ability of CXCL12/receptor interactions to induce secretion of chemical mediators such as ATP or NO from urothelial cells. A urothelial cell culture model where the cells could be stretched (in order to mimic distention) would best address these questions. Bioflex plates as part of the Flexcell FX-2000 unit (Flexcell International Corporation) are best suited for this purpose (Sun et al., 2001). In the presence of exogenously added CXCL12, we would hypothesize that serum collected from stretched urothelial cells would contain elevated levels of one or more mediators known to influence micturition function. Techniques to quantify released mediators include ELISA, western blot, luciferan-luciferase assays that measure ATP levels based on its catalytic effects during the oxidation reaction of luciferan to oxyluciferase plus light (Sun et al., 2001; Birder et al., 2002; Sun and Chai, 2006), and porphyrinic micro sensors detecting the oxidation of NO to NO⁺ to measure NO levels (Birder et al., 2001; Birder et al., 2002). The medium collected from the urothelial cells could then be applied to cultured afferent neurons in order to evaluate the ability of neurochemicals in the serum to modulate neuronal excitability. We would hypothesize that neuronal excitability, as measured by an increase in membrane potential or action potential frequency during intracellular current-clamp experiments (Tompkins et al., 2007), would dramatically increase after application of serum from CXCL12-treated urothelial cells.

The applied CXCL12 would, of course, be removed from the media prior to application of neuronal cultures. Utilizing immunoprecipitation techniques, an anti-CXCL12 antibody bound to agarose beads would absorb CXCL12 protein, and following centrifugation, agarose bound- CXCL12 will separate from the supernatant that can then be applied to neuronal cultures. To confirm that observed electrophysiological changes are due to CXCL12 signaling via the CXCR4 receptor, we would next block CXCR4 with AMD3100 in cultured urothelial cells prior to CXCL12 application. Theoretically, CXCR4 blockade would decrease CXCL12-induced urothelial release of neurochemicals and therefore serum applied to neurons from this instance should elicit a lesser response from cultured afferent neurons.

To assess the effect of CXCL12-mediated urothelial release on detrusor smooth muscle contractility, similar experiments could be performed *ex vivo* on bladder smooth muscle strips in place of afferent neurons. Excitability could be evaluated using intracellular recordings to measure membrane potential, action potential frequency and amplitude of afterhyperpolarization (Heppner et al., 1997). Additionally, myogenic experiments on bladder smooth muscle strips would support urothelial-mediated affects on detrusor contractility. We would first evaluate the contractility of the bladder in the presence of CXCL12 but with the urothelium removed. These experiments should demonstrate the ability, if any, of CXCL12 to influence detrusor contractility directly. CXCR4-IR was limited to the urothelium and so we would hypothesize that direct CXCL12 action on detrusor contractility does not exist or is very limited.

A second CXCL12 receptor, CXCR7, may contribute to direct CXCL12 effects on detrusor contractility. We did not evaluate CXCR7 protein expression in the urinary bladder, however quantitative PCR experiments demonstrate that detrusor mRNA transcript levels do not increase with 48 h CYP treatment in comparison to bladders from control rats. These data may suggest that CXCR7 does not contribute to CYP-induced bladder hyperreflexia observed with 48 h CYP treatment, the time point at which all cystometry experiments were conducted. To demonstrate the essential function of the urothelium, this experiment would be repeated with detrusor smooth muscle strips in the presence of an intact urothelium. We would predict that CXCL12 application in the presence of the urothelium should enhance the contractility of the smooth muscle due to chemokine-induced urothelial release of excitatory neurochemicals.

Summary and conclusions: pAKT signaling in the urinary bladder

We also investigated the contribution of an activated signaling molecule, phosphorylated (p) AKT to micturition under control and inflammatory conditions. In the bladder, pAKT has been shown to play a role in the invasiveness of tumor cells (Wu et al., 2004), LPS-induced infection (Tamarkin et al., 2006) and tissue remodeling following noxious pressure conditions (Stover and Nagatomi, 2007). Additionally, models of colonic inflammation (Qiao and Grider, 2009) and somatic peripheral injury or inflammation demonstrate pAKT involvement (Sun et al., 2006; Sun and Chai, 2006; Sun et al., 2007). We detected patchy, low-intensity basal pAKT expression in the urothelium of control bladders. Urothelial expression increases with 48 h CYP treatment, and this effect vanishes with chronic CYP-treatment, however, a suburothelial population of

pAKT-positive macrophages emerged at this time point. Staining of the detrusor smooth muscle reveals little to no pAKT expression in control bladders, however pAKT expression increases significantly with 48 h CYP treatment.

Again, conscious cystometry experiments combined with inhibitory pharmacological agents demonstrate the functional capacity of pAKT signaling in normal and inflamed micturition. Two different upstream inhibitors of AKT phosphorylation, AKT Inhibitor IV and Deguelin, independently increased bladder capacity, void volume and intercontraction interval following 48 h CYP-induced bladder inflammation. AKT Inhibitor IV only increased bladder capacity in control rats. It is possible that the role of AKT signaling in bladder function is less significant in non-inflamed micturition and may explain why Inhibitor IV, but not deguelin, affected micturition in control animals. In fact, somatic pain studies suggest that AKT signaling does not influence basal thermal or mechanical sensitivity prior to peripheral inflammation or injury (Sun et al., 2007; Xu et al., 2007). The present studies are consistent with previous reports where more dramatic effects on bladder function were observed in rats with CYP-induced bladder inflammation following intravesical delivery of inhibitors to AKT phosphorylation.

Future Directions: pAKT signaling in the urinary bladder

Future studies should examine potential downstream targets of AKT signaling. NGF signaling is known to activate the PI3-K/ AKT signaling pathway. In the urinary bladder CYP-induced activation of AKT is dependent on NGF expression (Chung et al., 2010). In addition, neurotrophic signaling molecules (PI3-K, MAPK, PLC) have been implicated in sensitization of TRPV1 in DRG neurons by enhancing calcium ion currents

(Bonnington and McNaughten, 2003; Galoyan et al., 2003; Zhu and Oxford, 2007).

These data were collected from afferent neurons, and although we did not detect any phosphorylated AKT in the suburothelial plexus nerve fibers, functional TRPV1 receptors are present in urothelial cells (Birder et al., 2001). TRPV1 channels are responsible for the majority of the inward cation current following stimulation with capsaicin (Caterina et al., 2000). Capsaicin application to cultured urothelial cells yields increasing intracellular calcium ion current and release of NO and ATP thus demonstrating functional sensory signaling capabilities of the urothelium via TRPV1 mediated mechanisms. Because neurotrophic signaling pathways play a role in TRPV1 sensitization in afferent neurons, activation of these same pathways in urothelial cells may produce similar effects.

An interesting next step would be, once again, to utilize a urothelial cell culture model to help elucidate a potential role for phosphorylated AKT activation in the sensitization of TRPV1-positive urothelial cells. Since capsaicin induces an intracellular calcium current in addition to the release of neurochemicals, we hypothesize that pretreatment with inhibitors of AKT phosphorylation would attenuate one or both of these effects. Additional studies employing inhibitors of other neurotrophic signaling pathways such as MAPK, JNK and PLC alone, or in combination, could help determine any cross-talk or synergistic effects of these pathways on TRPV1 sensitization.

Final conclusions

The data from my dissertation show that expression of the chemokine/ receptor pair CXCL12/CXCR4 and activation of the signaling molecule AKT increase in the

urinary bladder with inflammation, but not in suburothelial plexus nerve fibers.

Pharmacological blockade of CXCL12/CXCR4 signaling or AKT phosphorylation at the level of the urinary bladder rendered changes in micturition reflex function in both non-inflamed animals and those with CYP-induced bladder inflammation. Therefore plasticity that occurs within bladder tissue itself (i.e. non-neuronal) can influence micturition function, and therapeutic agents targeted to the bladder can improve inflammation-induced bladder hyperreflexia (Figure 1). Perhaps the strongest support for this concept is that intravesical blockade of CXCL12 signaling via the CXCR4 receptor antagonist AMD3100 in non-inflamed animals increases bladder capacity. This should not be surprising because basal CXCR4 expression is detected in the urothelium from control animals; however, the urothelium has the highest junction potential of any cell type recorded (Lewis and Diamond, 1976; Lewis, 2000) and therefore drug penetration through an intact urothelium (non-inflamed) is not likely. Therefore the mechanism of AMD3100-mediated increases in bladder capacity likely involves urothelial signaling, especially considering the localized presentation of the CXCR4 receptor.

Although we believe that pharmacological blockade at level of the urinary bladder is the primary source of improvements observed in these cystometrogram experiments, a role for signaling involved at the afferent level should not be entirely ruled out.

Immunohistochemical techniques are the most common way to determine neuronal expression in whole tissue preparations partially due to a lack of alternative methodologies, however antibody staining has a multitude of limitations and results should be analyzed with caution. We did not perceive any neuronal expression of the

proteins examined in these studies, even when using confocal imaging. This was surprising considering the numerous reports of chemokine and pAKT expression in sensory afferents (Tanaka et al., 2004; Qin et al., 2005; Bhangoo et al., 2007; Sun et al., 2007; Jung et al., 2008; Shi et al., 2009). It is possible that protein levels were too low to detect or that CXCL12 was secreted too quickly to be visualized with immunohistochemical techniques. Additionally, although the antibodies stained the urothelium and detrusor well, antibodies do not always stain different types of tissues with the same quality. Alternative primary antibodies were not used in the bladder whole mount preparations. Furthermore, visualization of CXCL12/CXCR4 or pAKT in the cell bodies of the primary afferent neurons was not attempted. Despite not being located in the terminals, somal expression could affect neuronal function.

It remains to be determined if an oral route of pharmacological blockade would be at least as beneficial as intravesical instillation when treating urinary bladder inflammation symptoms. This is especially important when considering IC/BPS in humans because the need for repeated intravesical instillations in a physician's office would be inconvenient, stressful and possibly painful. Additionally, evaluating micturition parameters with cystometry is somewhat limiting. During cystometry, the bladder is filling much more rapidly with saline than per physiological urine production, and the window of evaluation (1-2 h) does not offer insight to potential long-term therapeutic effects lasting days or weeks. Although we did not observe any wash-out effects of the drugs, we do not know how long therapeutic effects might last. Treatments

providing only a few hours of relief to patients living with chronic (lasting months to years) urgency, frequency and pain would not be sufficient.

Despite limitations, the present studies identify potential therapeutic targets that can be exploited, or at least tested to improve bladder function in the presence of inflammation. Key symptoms associated with CYP-induced bladder inflammation in rats are similar to those exhibited in patients with IC/ BPS such as bladder hyperreflexia and referred somatic pain. The anatomical origin of IC/ BPS symptoms is unknown and hotly debated as some argue that the bladder is the primary effector and yet others contest that pelvic floor muscles or other pelvic viscera via cross-sensitization contribute to both dysfunctional micturition and referred pain symptoms. The data from this dissertation provide further evidence for the efficacy of bladder targeted therapies to reduce inflammatory-induced hyperreflexia. However, the contribution of CXCL12/CXCR4 or AKT to CYP-induced referred somatic hypersensitivity was not assessed. Future studies examining the ability of intravesically infused agents to reduce CYP-induced somatic hyperalgesia would provide further evidence to the hypothesis that inflammatory-induced plasticity in the bladder itself can contribute to functional symptoms both in and beyond the urinary bladder.

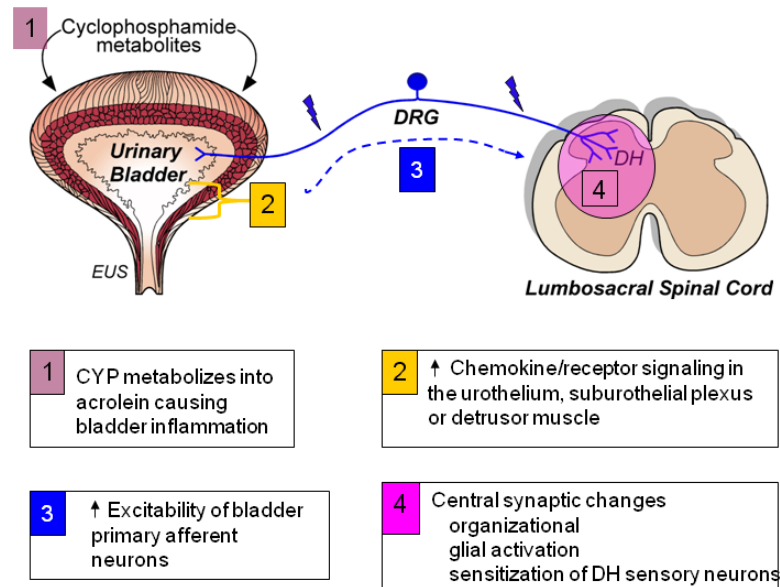


Fig. 1. Suggested areas of chemokine or pAKT action relative to IC/BPS symptoms.

- (1) CYP is metabolized to acrolein causing bladder inflammation
- (2) Chemokine or pAKT signaling increases in the urinary bladder.
- (3) Chemokine or pAKT signaling in the bladder indirectly sensitizes primary afferent via bladder mediated release of neurochemicals.
- (4) Intense activity of bladder afferent pathways leads to central synaptic changes potentiating central sensitization.

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Appendix A: Role of the Bladder Urothelium in Voiding Dysfunction

Arms, L., B.M. Girard, M.A. Vizzard. (2009). Role of the Bladder Urothelium in Voiding Dysfunction. *Current Bladder Dysfunction Reports*. 4: 227-233.

Abstract

The urothelium is a highly effective barrier acting as a constant permeability barrier protecting the blood from toxic urinary substances. However, a large and growing body of evidence now supports additional roles for the urothelium, including sensory and transduction/signaling roles enabling responses to chemical or mechanical stimuli. Reciprocal communication between urothelial cells with closely located bladder nerves and other cell types including detrusor smooth muscle and interstitial cells is likely. Diseases affecting the urinary bladder may affect urothelial receptor/channel expression and release of chemical mediators. Urothelial neuropeptide/receptor, chemokine/receptor, and neurotrophin/receptor expression, signaling/transduction and modulation in animal models of urinary bladder inflammation and human studies of bladder dysfunction are presented. Substrates underlying micturition reflex plasticity induced by urinary bladder disease/dysfunction are likely to include bladder primary afferent cells, lumbosacral spinal cord as well as the urothelium.

Introduction: Micturition Overview and Anatomy of Lower Urinary Tract

Micturition is regulated by neural circuits in the brain and spinal cord that coordinate the activity of the smooth and striated muscles of the lower urinary tract (LUT) [1]. These circuits act as on-off switches to shift the urinary tract between two modes of operation: storage and elimination. Bladder smooth muscle and the urethral outlet must function reciprocally for efficient elimination of urine [1]. The bladder smooth muscle must remain relaxed during storage mode to allow for filling, while the urethral outlet is contracted [1]. Elimination mode requires contraction of bladder smooth muscle and relaxation of the urethral outlet to allow urine flow¹. Precise coordination of the reciprocal functions of the urinary bladder and urethra and complex neural organization are required for normal function [1].

The LUT, consisting of the urinary bladder, urethra, internal urethral sphincter and external urethral sphincter, is composed of both striated and smooth muscle, and therefore under both voluntary (somatic) and involuntary (autonomic) influence [1]. The bladder consists of three layers: a urothelium on the luminal surface, a lamina propria just deep to the urothelium that contains a suburothelial plexus of nerves and vasculature, and an outer muscle layer, named the detrusor, that contains both longitudinal and circular smooth muscle [1]. The urothelium in rodents is composed of at least three layers: the basal, intermediate and superficial/umbrella layers. However, in higher mammals including humans, there are additional intermediate layers [2]. The umbrella cells are connected by tight junctions and are covered on their apical surface by crystalline proteins, which assemble into hexagonal plaques [2].

Urothelium: Much more than a passive protective barrier

The urothelium is a highly effective barrier acting as a constant permeability barrier protecting the blood from toxic urinary substances. The barrier function of the urothelium is dependent upon several features of the umbrella cell layer including formation of tight junction complexes and expression of specialized lipid molecules and uroplakins in the apical membrane [2]. The apical surface of the urothelium is also covered by a sulfated polysaccharide, glycosaminoglycan layer that is thought to act as an anti-adherence factor and defense mechanism against infection [3]. However, a large and growing body of evidence now supports additional roles for the urothelium, including sensory and transduction/signaling roles [4]. The current and emerging evidence suggests that the bladder urothelium not only acts as a functional barrier against urine solutes, but also exhibits specialized sensory and transduction/signaling properties enabling responses to chemical or mechanical stimuli [4, 5] (Table 1; Figure 1). Reciprocal communication between urothelial cells with closely located bladder nerves and other cell types including detrusor smooth muscle and interstitial cells is also a possibility based upon the anatomical substrates in place [4, 5] (Table 1; Figure 1). The terminology of an ‘urothelial-associated sensory web’ has been used to characterize the sensory inputs and sensory outputs of the urothelium that result in receptor- and channel-mediated events at the urothelium and subsequent release/secretion of chemical mediators [5] (Table 1; Figure 1).

“Neuron-Like” Properties of the Urothelium

Urothelial cells share a number of similarities with sensory neurons located in the dorsal root ganglia (DRG) resulting in the suggestion by Birder et al. [6-8] that the urothelium exhibits ‘neuron-like’ properties. Urothelial cells express a number of receptors and ion channels similar to those found in DRG sensory neurons [6, 9] (Table 1). The urothelial cells function as

transducers of some physical and chemical stimuli, secrete a variety of signaling molecules (e.g., neurotrophins, neuropeptides, ATP, acetylcholine, prostaglandins, prostacyclin, NO, cytokines) [4, 5] and communicate, perhaps reciprocally, with underlying cells including bladder nerves, smooth muscle, interstitial cells and inflammatory cells [10, 11] (Figure 1). It is this communication between urothelial cells and bladder nerves and subsequent input to the central nervous system in particular that may contribute to the physiology and pathology of bladder function and sensation.

As recently reviewed [12], there are several factors that are consistent with the suggestion that urothelial cells participate in the detection of physical and chemical stimuli.

Anatomical proximity of nerve fibers to the urothelium Recent studies have shown that both sensory afferent and autonomic efferent nerves are located in a suburothelial plexus in close proximity to the urothelium and extending into the urothelium although sensory afferent nerves are much more extensive in this plexus [13] (Figure 1). Studies have also demonstrated adrenergic (tyrosine hydroxylase) and cholinergic (choline acetyltransferase, ChAT) nerves in close proximity to the urothelium (for review see [12]). The suburothelial plexus exhibits immunoreactivity for neuropeptides (e.g., CGRP, substance P, CRH), purinergic receptors (P2X-) and transient receptor potential vanilloid receptor 1 (TRPV1) [14, 15]. Intravesical administration of the ultrapotent C-fiber neurotoxin, resiniferatoxin, reduced the density of TRPV1 and P2X3 immunoreactive suburothelial nerves in humans with neurogenic detrusor overactivity, indicating the sensory origin of these nerve fibers [16, 17].

Urothelial expression of receptors/ion channels Urothelial cells express numerous receptors/ion channels (Table 1) that are linked to mechanosensitive or nociceptive sensations. Numerous

receptors/ion channels [4, 5] have been identified in the urothelium including, purinergic receptors (e.g., P2X₁₋₇ and P2Y_{1,2,4}), norepinephrine (α and β), acetylcholine (muscarinic and nicotinic), protease-activated receptors (PARs), acid sensing ion channels (e.g., ASIC2a, ASIC3) [Corrow and Vizzard, unpublished], neurotrophin receptors (p75^{NTR}, TrkA, TrkB) [9, 18], corticotropin releasing factor receptors (CRHR1 and CRHR2) [15], TRP channels, [4, 5, 19-21], neuropeptide receptors (e.g., PAC1, VPAC2) [22, 23], and chemokine receptors (e.g., CXCR4, CX3CR1) [24] (Table 1). With expression of these receptors/ion channels (Table 1), the urothelium can respond to diverse inputs from multiple sources. These inputs include increased stretch/distention during the bladder filling phase, soluble factors such as nerve growth factor (NGF), neuroactive compounds such as pituitary adenylate cyclase activating polypeptide (PACAP), vasoactive intestinal polypeptide (VIP), corticotropin releasing factor (CRH), acetylcholine, or norepinephrine released from nerves, inflammatory cells [4, 5, 23-25] or chemokines released from inflammatory cells and changes in pH resulting from inflammation [26-29].

Urothelial secretion of transmitters and mediators The urothelium secretes a variety of signaling molecules (e.g., neurotrophins, neuropeptides, ATP, acetylcholine, prostaglandins, prostacyclin, NO, cytokines) [4, 5] (Figure 1) and is able to communicate, perhaps reciprocally, with underlying cells including bladder nerves, smooth muscle, interstitial cells and inflammatory cells [4, 5] (Figure 1). Urothelial-derived NO released in response to mechanical as well as chemical stimulation can either facilitate or inhibit the activity of bladder afferent nerves [4]. Additional studies demonstrate that the urothelium can modulate the spontaneous activity of the underlying smooth muscle through release of various soluble factors [10, 11].

Modification of the urothelium with injury or inflammation

Although highly effective in maintaining an effective barrier, there are many ways in which the integrity of the urothelium can be compromised including mechanical or chemical trauma, changes in tissue pH often associated with infection or inflammation, or bacterial infection itself [4]. The clinical syndrome of interstitial cystitis (IC)/painful bladder syndrome (PBS) is also associated with a compromise in urothelium integrity that can result in water, urea and toxic substances passing into underlying neural and smooth muscle tissue layers [30, 31]. A compromised urothelial barrier can produce the symptoms of urgency, frequency and pain during the filling and elimination phases of the micturition cycle.

The Clinical Syndrome: Interstitial Cystitis (IC)/Painful Bladder Syndrome (PBS)

IC/PBS is a chronic inflammatory bladder disease syndrome characterized by urinary frequency, urgency, suprapubic and pelvic pain [32, 33]. Numerous theories including infection, autoimmune disorder, toxic urinary agents, deficiency in bladder wall lining, neurogenic causes and urothelial dysfunction have each been proposed as the underlying cause(s) of IC/PBS. Pain and altered bladder function in IC/PBS may involve a change in visceral sensation/bladder sensory physiology. Altered bladder sensations [32, 33] may be mediated by changes in peripheral bladder afferent and central pathways as well as by changes in urothelial signaling to bladder nerves in the suburothelial plexus such that bladder afferent neurons respond in an exaggerated manner to normally innocuous stimuli (i.e., allodynia) or exhibit heightened responses to noxious stimuli (i.e., hyperalgesia).

In the following sections, we will discuss some examples of receptors expressed in the urothelium (Table 1), responses upon activation and regulation of receptors and signaling

properties in animal models of bladder dysfunction and in human studies of bladder dysfunction where appropriate. Although our focus in this chapter is to address some sensory properties (ion channels/receptors) and signaling/transduction properties of the urothelium that have not received substantial attention previously, we first describe distension-evoked release of ATP [20] from the urothelium as a well-characterized and seminal description of the secretory properties of the urothelium and subsequent identification of purinergic receptor expression [34] and modulation with bladder dysfunction [19, 35]. Many other sensory and transduction properties of the urothelium have been recently reviewed [12].

Role of receptors/ion channels and secretory products in urothelial function

Urothelium and purinergic receptor/signaling

From the initial report of ATP release from the urothelium upon stretch [20], it was apparent that the urothelium could respond to stimuli and secrete a signaling molecule, ATP (Figure 1). ATP signals via ligand-gated ion purinergic receptors, specifically the P2X family. Many sensory neurons terminating in the suburothelial plexus of the bladder as well as urothelial cells densely express a member of the P2X family, P2X₃ [36]. In response to stretch, urothelial cells release ATP, which can then act on P2X₃ receptors in bladder sensory neurons. Altered ATP purinergic signaling in the bladder may provide a platform for alterations in sensory mechanisms associated with IC/PBS. Interestingly, urine of IC/PBS patients contains elevated levels of ATP. Further, bladders of IC/PBS patients show enhanced ATP release from urothelial cells in response to stretch [35], and increased expression of P2X₃ receptors in urothelial cells [34] possibly enhancing purinergic signaling. Increased ATP release from urothelial cells

combined with increased density of P2X₃ receptors may lead to mechanical hypersensitivity and pain.

Urothelium and neurotrophin/receptor signaling

A variety of local and distant events can damage the urothelium. Injury accelerates and induces both proliferation and differentiation to restore the urothelial barrier. The initiation of urothelial proliferation is thought to involve upregulation of growth factors such as nerve growth factor (NGF). NGF belongs to the neurotrophin superfamily, which also includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5 and NT-6 [37]. All neurotrophins bind with low affinity to a membrane spanning receptor molecule, known as the low-affinity NGF receptor (p75^{NTR}), a member of the tumor necrosis factor (TNF- α) receptor family [37]. The trophic effect of all neurotrophins, including NGF, requires binding to recognition molecules of the tyrosine kinase (Trk) family of receptors, activated in response to neurotrophin binding, although Trk-independent signal transduction through p75^{NTR} can also occur [37]. The urothelium expresses p75^{NTR}, TrkA and TrkB and expression is modulated with bladder inflammation induced by cyclophosphamide (CYP) [9, 18] (Figure 1). Intracellular signaling cascades activated by Trk receptors include the Ras/ERK pathway, the phosphatidylinositol-3-kinase (PI3K)/Akt kinase pathway as well as the phospholipase C α (PLC α) cascade. Recent studies have demonstrated the involvement of the ERK1/2, ERK5 and PI3K/Akt kinase pathways in CYP-induced cystitis with blockade reducing urinary bladder hyperreflexia [37] [Arms and Vizzard, unpublished].

Although the exact contribution of NGF to bladder function is not clear, NGF seems to play a role in bladder hyperreflexia or bladder overactivity. NGF upregulation occurs at sites of tissue injury and inflammation and changes in NGF levels in urine as well as urinary bladder have been documented in humans with IC/PBS and rodents with bladder inflammation [37, 38]. Clinically, elevated levels of neurotrophins have also been detected in the urine of women with IC/PBS [39] as well as in the urothelium of individuals with painful bladder conditions [40]. More recently, it has been demonstrated that urinary NGF levels are increased in patients with overactive bladder (OAB) and urinary incontinence and decreased in patients responding to botulinum toxin-A treatment [41, 42]. The role(s) of NGF in micturition reflexes and sensation have been evaluated using a variety of addition and subtraction techniques (for review see [37]).

Neurotrophins, through interactions with Trk and/or $p75^{\text{NTR}}$ receptors, may contribute to neural plasticity of micturition pathways with bladder inflammation and to bladder dysfunction. Intravesical instillation of k252a, a pan Trk inhibitor, in CYP-treated rats reduces bladder hyperreflexia [37]. In contrast, recent studies demonstrate bladder hyperreflexia or enhanced hyperreflexia in control or CYP-treated rats following intravesical blockade of $p75^{\text{NTR}}$ [43]. Thus, one function of $p75^{\text{NTR}}$ and NGF- $p75^{\text{NTR}}$ interactions in vivo may be to reduce bladder activity or to offset bladder hyperreflexia induced by CYP-induced cystitis [43]. Whereas $p75^{\text{NTR}}$ expression can enhance TrkA-NGF binding in some systems [37], $p75^{\text{NTR}}$ may also reduce NGF-TrkA signaling by sequestering NGF [43]. Any decrease or perturbation in NGF/ $p75^{\text{NTR}}$ binding may tip the homeostatic equilibrium, resulting in increased NGF bioavailability to enhance NGF/TrkA signaling. If NGF/TrkA signaling is favored, increased expression or

function of ion channels such as the transient receptor potential (TRP) channels (e.g., TRPV1) and neuropeptides may contribute to urinary bladder hyperreflexia.

A transgenic mouse model of chronic NGF overexpression (OE) in the bladder

To further explore the role of NGF in bladder sensory function, a transgenic mouse model of chronic NGF OE in the bladder using the urothelial-specific uroplakin II (UPII) promoter was generated [38]. The NGF OE mice were generated at Roche Palo Alto by Dr. Debra Cockayne in collaboration with Dr. Henry Sun at New York University Medical School. NGF was overexpressed at both the mRNA and protein level in the bladders of these transgenics compared to WT littermate controls. Conscious open voiding cystometry studies demonstrated that transgenic mice had a reduced bladder capacity and an increase in the number and amplitude of non-voiding bladder contractions under baseline conditions. These changes in bladder function were further associated with an increased referred somatic hypersensitivity in the pelvic region. NGF OE mice may provide a novel transgenic model for further exploring the role of NGF in urinary bladder dysfunction [38].

Urothelium and PACAP/VIP and associated receptors signaling

A number of peptides have been established in the LUT and have demonstrated roles in regulating the micturition reflex. PACAP (pituitary adenylate cyclase activating polypeptide) and VIP (vasoactive intestinal polypeptide) are members of the glucagon/secretin superfamily of hormones [44]. PACAP and VIP share receptor subtypes coupled to different intracellular effectors; PACAP peptides exhibit high affinity for the PAC1 receptor, whereas VIP and PACAP have similar high affinities for the VPAC1 and VPAC2 receptors [44]. Both PACAP- and VIP-

immunoreactivity (IR) have been identified in urinary bladder [22, 45]. Widespread PACAP-IR exists in nerve fibers in rat LUT with the majority of the PACAP nerve fibers being derived from sensory neurons [45]. PACAP receptors have been identified in various tissues of the micturition pathway including bladder detrusor smooth muscle and urothelium (Table 1; Figure 1).

PACAP/receptors and micturition

Recent studies support roles for PACAP in micturition and suggest that inflammation-induced plasticity in PACAP expression in peripheral and central micturition pathways contribute to bladder dysfunction with cystitis. We have previously demonstrated facilitatory direct effects of PACAP on bladder smooth muscle contractility [22]. PACAP increased bladder smooth muscle tone and potentiated electric field stimulation (EFS)-induced contractions [22]. EFS-induced contractions were superimposed on spontaneous muscle contractions and were tetrodotoxin-insensitive suggesting that the responses were direct detrusor smooth muscle effects [22]. In a rat CYP-induced cystitis paradigm, intrathecal or intravesical administration of PAC1 receptor antagonist, PACAP6-38, reduced cystitis-induced bladder hyperreflexia.

CYP-induced cystitis With acute CYP-induced cystitis, PAC1 receptor transcript exhibited a significant decrease in expression in both urothelium and detrusor; however, a significant increase in PAC1 receptor transcript expression in urothelium and detrusor smooth muscle was induced by intermediate and chronic CYP-induced cystitis [23]. PACAP transcript expression significantly increased in the urothelium with intermediate (48 hr) and chronic (8 days) CYP treatment whereas no changes were observed with acute (4 hr) CYP-induced cystitis in either urothelium or detrusor smooth muscle [23].

VIP/receptors and micturition

VIP exerts species-specific, excitatory or inhibitory actions in neural pathways controlling micturition and these functions may be altered with neural injury, disease or inflammation. A number of diverse and conflicting roles for VIP have been demonstrated in the urinary bladder from numerous species. These contradictory findings might be attributable to species differences [46] and differential VIP receptor distribution. Surprisingly, VIP had no apparent effects on either bladder tone or EFS-stimulated contractions despite VPAC2 receptor transcript expression in detrusor [22]. However, as VIP innervation to the urinary bladder is minimal compared to that for PACAP [45], these results may be in keeping with suggestions that PACAP and PAC1 signaling are more prominent regulators of rat bladder physiology [22]. These VIP results are consistent with previous studies that demonstrated that VIP application to detrusor smooth muscle had no effect on spontaneous or carbachol-induced bladder contractions despite facilitation of micturition when VIP was administered intrathecally or intraarterially close to the rat bladder [47]. Recent studies [48] using VIP^{-/-} mice reveal that VIP^{-/-} mice exhibit increased bladder mass and fewer but larger urine spots on filter paper. VIP^{-/-} mice display increased void volumes and shorter intercontraction intervals with continuous intravesical infusion of saline. No differences in transepithelial resistance or water permeability were demonstrated between VIP^{-/-} and WT mice. With the induction of bladder inflammation by acute administration of CYP, an exaggerated or prolonged bladder hyperreflexia was demonstrated in VIP^{-/-} mice [48]. The changes in bladder hyperreflexia may reflect increased expression of neurotrophins and/or or proinflammatory cytokines in the urinary bladder.

CYP-induced cystitis in rat No regulation of VIP transcript expression was detected at any duration of CYP treatment in urothelium or detrusor [23]. CYP-induced changes in VPAC1 and VPAC2 expression were similar in both urothelium and detrusor. Acute and intermediate CYP-induced cystitis increased VPAC1 and VPAC2 receptor transcript expression in both urothelium and detrusor. In contrast, chronic CYP induced cystitis decreased VPAC2 receptor transcript expression in both urothelium and detrusor [23]. Thus, these cystitis-induced changes may further regulate the neural control of micturition.

PACAP-mediated ATP release from cultured, rat urothelium

Earlier studies have indicated that the urothelium releases ATP in response to various stimuli [49]. In addition, it has been suggested that ATP released from the serosal surface of the urothelium during bladder filling stimulates receptors on suburothelial sensory nerve fibers and contributes to bladder filling sensation [36] (Figure 1). PACAP and VIP application evoked ATP release from rat urothelial cell cultures; however, ATP release was greatest with PACAP treatment and significantly blocked by the PAC1 receptor selective antagonist, M65 [23]. The current data further support the suggestion that PACAP and PAC1 signaling are regulators of bladder physiology at the level of the urinary bladder and specifically, the urothelium.

Urothelium and chemokine/receptor systems signaling

Chemokines, chemotactic cytokines, are a large family of structurally and functionally related proteins that are important mediators of the immune response, nociception and inflammatory processes. For example, in a model of chemically induced-bladder inflammation, CYP treatment increases the urothelial expression of certain chemokines and their receptors such as fractalkine (CX3CL1) and its receptor CX3CR1 [24], and CXCL12 and its receptor CXCR4

[26] (Table 1; Figure 1). Receptor blockade of CXCR4 reduces CYP-induced bladder hyperexcitability as demonstrated by significant increases in intercontraction interval, bladder capacity and void volume [28]. Additionally, blockade of CXCL10 reduces CYP-induced symptoms such as urinary bladder leukocyte infiltration, hyperplasia and epithelial erosions [27].

The combined CYP-induced expressional disparity and functional changes following signaling blockade indicate that chemokine signaling may play a role in altered sensory processing of the urinary tract. Recent studies suggest that chemokines secreted by their host cells may signal in a paracrine fashion by binding to their cognate receptors on neighboring sensory neurons and thus facilitating hyperexcitability [50] by changing membrane potentials, decreasing thresholds for action potential generation, modulating calcium ion currents and increasing excitability and evoking discharges [29, 50]. Alternatively, chemokines may signal in an autocrine fashion by binding firstly to their cognate receptors on their host cells (e.g. urothelial cells) that then communicate to the nervous system secondly. In either paradigm, the proximity of the urothelial cells to the dense layer of sensory nerve terminals in the suburothelial plexus beneath provides a potential mechanism for chemokines produced in the bladder to induce functional sensory changes.

Conclusions

IC/PBS is characterized by suprapubic and pelvic pain, urinary frequency and urgency. Although the underlying etiology of IC/PBS is not known, several factors, alone or in combination, may contribute to the bladder and sensory dysfunction including neurogenic inflammation, autoimmune involvement and urothelial dysfunction. Given the current evidence for sensory and transduction/signaling abilities of the urothelium and the ability of the

urothelium to influence adjacent tissues including the suburothelial nerve plexus, myofibroblasts and detrusor smooth muscle cells, it is likely that changes in the sensory or barrier functions of the urothelium contribute to bladder and sensory dysfunction in bladder diseases including IC/PBS. Given the ability of diverse stimuli to influence urothelial release of chemical mediators (e.g., neurotrophins, neuropeptides, ATP, acetylcholine, prostaglandins, prostacyclin, NO, cytokines) and for receptor/channel expression and release properties to be altered with bladder disease, the urothelial receptors/channels represent novel targets for potential treatments/therapies.

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Table 1: Sensor and receptor properties of urinary bladder urothelial cells

Table 1 Sensor and receptor properties of urinary bladder urothelial cells	
Sensor stimuli	Urothelial Receptor Expression
Acetylcholine	Nicotinic and muscarinic receptors
ATP	Purinergic receptors (P2X, P2Y)
Bradykinin	B1 and B2 bradykinin receptors
Capsaicin and resiniferatoxin	TRPV1
Chemokines (e.g., CX3CL1, CXCL12)	CX3CR1, CXCR4
Cold	TRPM8, TRPA1
Corticotropin releasing factor	CRHR1, CRHR2
H ⁺	TRPV1
H ⁺ , cold	ASIC
Heat	TRPV1, TRPV2, TRPV4
Moderate heat, osmolarity	TRPV4
Nerve growth factor	p75 ^{NTR} , TrkA
Norepinephrine	α and β adrenergic receptors
VIP, PACAP	PAC1, VPAC1, VPAC2

Abbreviations: ATP, adenosine triphosphate; H⁺, protons; TRPA, transient receptor potential

ankyrin-type channel; TRPM, transient receptor potential melastatin-type channel; TRPV,

transient receptor potential vanilloid-type channel; TrkA, receptor tyrosine kinase A; p75^{NTR},

low affinity neurotrophin receptor; CRHR1, corticotropin releasing factor receptor 1; CRHR2,

corticotropin releasing factor receptor 2; VIP, vasoactive intestinal polypeptide; PACAP,

pituitary adenylate cyclase activating polypeptide; ASIC, acid-sensing ion channel.

Figure Legend

Figure 1: Potential model of possible reciprocal interactions among bladder afferent and efferent nerves (not shown), urothelial cells, myofibroblasts located in the suburothelium and detrusor smooth muscle that underlie physiological bladder reflex function as well as pathophysiology in bladder disease. Receptor activation and channel stimulation on urothelial cells can elicit secretion of chemical mediators that can affect adjacent tissues including bladder nerves in the suburothelial plexus, myofibroblasts and detrusor smooth muscle. Urothelial cells can also be responsive to neurotransmitters released from bladder nerves and other cell types including inflammatory cells. Abbreviations: ATP, adenosine triphosphate; TRPs, transient receptor potential channels; TrkA, receptor tyrosine kinase A; TrkB, receptor tyrosine kinase B; p75^{NTR}, low affinity neurotrophin receptor; CRHR2, corticotropin releasing factor receptor 2; ASIC, acid-sensing ion channel; PG, prostaglandin; NO, nitric oxide; NGF, nerve growth factor; NP, neuropeptides; Ach, acetylcholine; PAC1, pituitary adenylate cyclase-activating polypeptide (PACAP) selective receptor; VPAC2, receptor with equal and high affinity for vasoactive intestinal polypeptide and PACAP. See text for additional details. Figure modified from [4].

Figure

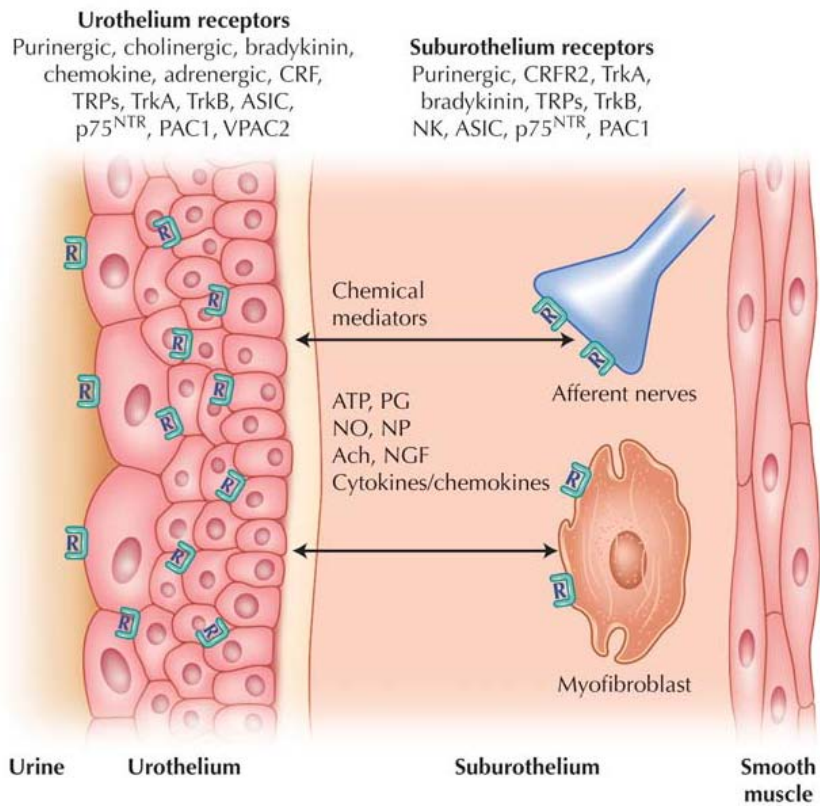


Figure 1

Appendix B: Neuropeptides in Lower Urinary Tract (LUT) Function

Arms, L., B.M. Vizzard. (2010). Neuropeptides in lower urinary tract (LUT) function. Chapert 19 in the *Handbook of Experimental Pharmacology. Pharmacology of the lower urinary tract*. In Press.

Abbreviations

AA	acetic acid
ATP	adenosine triphosphate
BOO	bladder outlet obstruction
CGRP	calcitonin gene-related peptide
CYP	cyclophosphamide
DRG	dorsal root ganglia
DSD	detrusor sphincter dyssynergia
EFS	electric field stimulation
hr	hours
IC	interstitial cystitis
ICI	intercontraction interval
IL	interleukin
IR	immunoreactivity
LUT	lower urinary tract
NGF	nerve growth factor
NK	neurokinin
NVCs	non-voiding contractions
OAB	overactive bladder
PACAP	pituitary adenylate cyclase activating polypeptide
PBS	painful bladder syndrome
SCI	spinal cord injury
SP	substance P

VIP	vasoactive intestinal polypeptide
VV	void volume
WT	wildtype

Abstract

Numerous neuropeptide/receptor systems including vasoactive intestinal polypeptide, pituitary adenylate cyclase-activating polypeptide, calcitonin gene-related peptide, substance P, neurokinin A, bradykinin, and endothelin-1 are expressed in the lower urinary tract (LUT) in both neural and non-neural (e.g., urothelium) components. LUT neuropeptide immunoreactivity is present in afferent and autonomic efferent neurons innervating the bladder and urethra and in the urothelium of the urinary bladder. Neuropeptides have tissue-specific distributions and functions in the LUT and exhibit neuroplastic changes in expression and function with LUT dysfunction following neural injury, inflammation and disease. LUT dysfunction with abnormal voiding including urinary urgency, increased voiding frequency, nocturia, urinary incontinence and pain may reflect a change in the balance of neuropeptides in bladder reflex pathways. LUT neuropeptide/receptor systems may represent potential targets for therapeutic intervention.

Keywords

substance P, calcitonin gene-related peptide, vasoactive intestinal polypeptide, pituitary adenylate cyclase activating polypeptide, nerve growth factor

19.1 Introduction: Micturition Overview and Anatomy of Lower Urinary Tract

Micturition is regulated by neural circuits in the brain and spinal cord that coordinate the activity of the smooth and striated muscles of the lower urinary tract (LUT) (Fowler et al., 2008). These circuits act as on-off switches to shift the urinary tract between two modes of operation: storage and elimination. Bladder smooth muscle and the urethral outlet must function reciprocally for efficient elimination of urine (Fowler et al., 2008). The bladder smooth muscle must remain relaxed during storage mode to allow for filling, while the urethral outlet is contracted (Fowler et al., 2008). Elimination mode requires contraction of bladder smooth muscle and relaxation of the urethral outlet to allow urine flow (Andersson and Arner, 2004). Precise coordination of the reciprocal functions of the urinary bladder and urethra and complex neural organization are required for normal function (Fowler et al., 2008).

The LUT, consisting of the urinary bladder, urethra, internal urethral sphincter and external urethral sphincter, is composed of both striated and smooth muscle, and therefore under both voluntary (somatic) and involuntary (autonomic) influence (Andersson and Arner, 2004). The bladder consists of three layers: a urothelium on the luminal surface, a lamina propria just deep to the urothelium that contains a suburothelial plexus of nerves and vasculature, and an outer muscle layer, named the detrusor, that contains both longitudinal and circular smooth muscle (Andersson and Arner, 2004) (Fig. 19.1). The urothelium in rodents is composed of at least three layers: the basal, intermediate and superficial/umbrella layers. However, in higher mammals including humans, there are additional intermediate layers (Wu et al., 2009). The umbrella cells are

connected by tight junctions and are covered on their apical surface by crystalline proteins, which assemble into hexagonal plaques (for review see (Wu et al., 2009)).

19.1.1 Neural control of micturition

The storage and periodic elimination of urine requires a complex neural control system that coordinates the activities of a variety of effector organs including the smooth muscle of the urinary bladder and the smooth and striated muscle of the urethral sphincters (Kuru, 1965; Klück, 1980; de Groat and Steers, 1990; Andersson and Arner, 2004). Three neural pathways regulate the LUT: (1) sacral parasympathetic (pelvic) nerves provide excitatory input to the bladder; (2) thoracolumbar sympathetic (hypogastric) nerves provide an inhibitory input to the bladder and an excitatory input to the bladder neck and urethra; and (3) sacral somatic (pudendal) nerves which innervate the striated muscles of the sphincters and pelvic floor (Kuru, 1965; Klück, 1980; de Groat and Steers, 1990; Middleton and Keast, 2004). Each of these sets of nerves contains afferent (sensory) as well as efferent (motor) axons (Morrison, 1987; Lincoln and Burnstock, 1993).

The central neural pathways controlling the LUT exhibit "all-or-none" or "switch-like" characteristics reflecting the storage and elimination functions of the LUT (de Groat et al., 1993; de Groat and Kruse, 1993; de Groat, 1997). During urine storage, somatic and sympathetic pathways to the sphincters and sympathetic inhibitory inputs to the bladder are tonically active whereas parasympathetic pathways are inactive (Kuru, 1965; de Groat et al., 1993; de Groat and Kruse, 1993; de Groat, 1997). During reflex or voluntary micturition, the activity patterns are reversed such that parasympathetic

pathways are excited and somatosympathetic pathways are inhibited thereby promoting urine flow (Middleton and Keast, 2004).

The LUT reflex mechanisms, organized at the level of the lumbosacral spinal cord, are modulated predominantly by supraspinal controls (Kuru, 1965; de Groat, 1975; de Groat et al., 1993; de Groat and Kruse, 1993; de Groat, 1997; Middleton and Keast, 2004). These mechanisms can be summarized as follows: (1) storage reflexes (parasympathetic and somatic) are organized at the spinal level; (2) elimination reflexes (parasympathetic) are organized at a supraspinal site in the pons; and (3) spinal storage reflexes are modulated by inputs from the rostral pons.

19.1.2 Neurochemistry and morphology of afferent and spinal pathways to the urogenital tract

Bladder afferent neurons travel in the hypogastric and pelvic nerves, and their cell bodies are located in dorsal root ganglia (DRG) at spinal segments T11-L2 and S2-S4 in humans and L1-L2 and L6-S1 in rats (Fowler et al., 2008) (Fig. 19.1). Bladder afferent fibers consist of lightly myelinated A δ fibers and unmyelinated C-fibers. Sensation of bladder filling is conveyed by A δ fibers, the most important mechanoreceptors of the bladder. C-fibers are normally “silent”, but they do respond to chemical or noxious stimuli, including extreme bladder pressure (Fowler et al., 2008). A δ and C-fibers terminate in the urothelium, suburothelium, and smooth muscle layers of the bladder (Kullmann et al., 2008). Most bladder afferent fibers project to lumbosacral spinal cord segments, and this is the most important region of the spinal cord relative to signaling the micturition reflex (Holstege, 2005) (Fig. 19.1). Most sensory nerves in the bladder are

located in a dense suburothelial plexus just beneath the urothelium (Andersson and Wein, 2004) (Fig. 19.1).

Bladder afferent fibers in the pelvic nerve in rodents pass through the dorsal roots into Lissauer's tract at the apex of the dorsal horn and then give off collateral branches that extend ventromedially and ventrolaterally along the superficial layers of the dorsal horn to the dorsal commissure and to the area of the sacral parasympathetic nucleus (laminae V-VII) that contains preganglionic parasympathetic neurons that project to the periphery (de Groat et al., 1981; Donovan et al., 1983; de Groat et al., 1986; Steers et al., 1991a; de Groat and Kruse, 1993; de Groat et al., 1994; Nadelhaft and Vera, 1995; de Groat, 1997; Marson, 1997). The most prominent pathway is located in lamina I on the lateral edge of the dorsal horn in a region termed the lateral collateral pathway of Lissauer's tract. Afferent projections from the pudendal nerve and genital structures follow the medial edge of the dorsal horn into the dorsal commissure region, forming the medial collateral pathway (Kawatani et al., 1990). Bladder afferent fibers contain a variety of neuropeptides, including: calcitonin-gene related peptide (CGRP), substance P (SP), neurokinin A, neurokinin B, vasoactive intestinal polypeptide (VIP), cholecystokinin and enkephalins (Donovan et al., 1983; de Groat et al., 1986; Keast, 1991; de Groat et al., 1996; Vizzard, 2000d; Vizzard, 2001) (Fig. 19.1). We have demonstrated (Vizzard, 2000d) that bladder afferent cells express pituitary adenylate cyclase-activating polypeptide (PACAP) and that expression is increased after cyclophosphamide (CYP)-induced cystitis in rats. With the exception of CGRP, all of these substances are predominantly expressed in small (presumably C-fiber) afferents (Ek

et al., 1977; Donovan et al., 1983; de Groat et al., 1986; Su et al., 1986; Keast and de Groat, 1992; Vizzard et al., 1993a; Vizzard et al., 1993b; Vizzard et al., 1994; Vizzard et al., 1995; de Groat et al., 1996; Vizzard and de Groat, 1996). The administration of capsaicin, which acts selectively on small diameter afferent fibers to deplete neurotransmitter stores to induce neuronal degeneration, reduces the levels of substance P, neurokinin A and CGRP within the pelvic viscera but does not affect VIP or enkephalin expression (de Groat, 1987). These findings are consistent with SP, related tachykinins and CGRP expression in afferent pathways to the pelvic viscera (de Groat, 1987).

Many bladder afferent fibers project to the sacral parasympathetic nucleus, synapsing with preganglionic parasympathetic neurons as well as interneurons (Morgan et al., 1981; Fowler et al., 2008). Primary bladder afferents from the pelvic and hypogastric nerves also project to the dorsal commissure and superficial dorsal horn (Fowler et al., 2008). The lumbosacral dorsal commissure, superficial dorsal horn, and parasympathetic nucleus all contain interneurons important to urinary bladder function (de Groat and Kruse, 1993; Fowler et al., 2008). These interneurons project locally in the spinal cord or to the brain (Fowler et al., 2008). Some bladder afferents synapse with ascending pathways in the spinal cord that project to neuronal populations in the brain involved in micturition control, including the pontine micturition center (de Groat and Kruse, 1993; Fowler et al., 2008).

19.1.3 Neurochemical plasticity in the LUT with bladder inflammation, neural injury or disease

Neuroactive compounds, including neuropeptides, in the afferent pathways to the LUT exhibit either excitatory or inhibitory actions. Non-neural sources of peptides in the LUT include plasma, sites of tissue inflammation or injury, detrusor smooth muscle cells, bladder fibroblasts and the urothelium. Pathology, neural injury and target organ pathology (e.g., bladder inflammation) can alter the known balance of neuropeptides either in the periphery and/or central pathways conceivably shifting the balance to a hyper- or hypo-active reflex state. Changes in micturition reflex function observed with urinary bladder inflammation (Vizzard, 2000d; Vizzard, 2000b; Vizzard, 2001), interstitial cystitis (IC)/painful bladder syndrome (PBS), spinal cord injury (upper motoneuron injury) (Vizzard, 2006; de Groat and Yoshimura, 2009), overactive bladder (OAB) (Yoshimura et al., 2008), and detrusor overactivity secondary to bladder outlet obstruction (BOO) (Andersson, 1999; 2006) may reflect a change in the balance of neuropeptides in LUT reflex pathways. Information presented in this review will address both direct and indirect effects of neuropeptides in the LUT. Due to neuropeptide/receptor expression and diversity of functions in the LUT and subsequent regulation with neural injury, disease, and bladder inflammation, neuropeptide/receptor systems may be potential targets for therapeutic intervention (Fig. 19.1).

The following sections will address the distribution, function and regulation of specific neuropeptide/receptor systems in the LUT under normal and pathological LUT conditions.

19.2 PACAP/VIP and associated receptors signaling in the LUT

A number of peptides have been demonstrated in the LUT and have demonstrated roles in regulating the micturition reflex. PACAP and VIP are members of the glucagon/secretin superfamily of hormones (Dickinson et al., 1999). PACAP and VIP share receptor subtypes coupled to different intracellular effectors; PACAP peptides exhibit high affinity for the PAC1 receptor, whereas VIP and PACAP have similar high affinities for the VPAC1 and VPAC2 receptors (Arimura, 1998; Sherwood et al., 2000). Both PACAP- and VIP-immunoreactivity (IR) have been identified in urinary bladder (Fahrenkrug and Hannibal, 1998; Mohammed et al., 2002). Widespread PACAP-IR exists in nerve fibers in rat LUT with the majority of the PACAP nerve fibers being derived from sensory neurons (Fahrenkrug and Hannibal, 1998; Zvarova et al., 2005). PACAP receptors have been identified in various tissues of the micturition pathway including bladder detrusor smooth muscle, urothelium and major pelvic ganglia (Table 19.1)(Braas et al., 2006; Tompkins et al., 2010).

19.2.1 PACAP/receptors in micturition reflex pathways

Recent studies support roles for PACAP in micturition and suggest that inflammation-induced plasticity in PACAP expression in peripheral and central micturition pathways contribute to bladder dysfunction with cystitis. We have previously demonstrated facilitatory direct effects of PACAP on bladder smooth muscle contractility (Braas et al., 2006). PACAP increased bladder smooth muscle tone and potentiated

electric field stimulation (EFS)-induced contractions (Braas et al., 2006). EFS-induced contractions were superimposed on spontaneous muscle contractions and were tetrodotoxin-insensitive suggesting that the responses were direct detrusor smooth muscle effects (Braas et al., 2006). Excitatory effects of PACAP on the micturition reflex pathway are enhanced 2-4 weeks after spinal cord injury in the rat (Yoshiyama and de Groat, 1997).

19.2.2 PACAP expression in bladder afferent pathways and regulation by CYP-induced cystitis

PACAP is expressed in LUT pathways and is regulated by CYP-induced cystitis (Vizzard, 2000d; Braas et al., 2006). In control rats, PACAP-IR was expressed in fibers in the superficial dorsal horn at all segmental levels examined (L1, L2, and L4 –S1). Bladder afferent cells (40–45%) in the dorsal root ganglia (DRG; L1, L2, L6, and S1) from control animals also exhibited PACAP-IR (Vizzard, 2000d). After chronic, CYP-induced cystitis, PACAP-IR increased dramatically in spinal segments and DRG (L1, L2, L6, and S1) involved in micturition reflexes (Vizzard, 2000d). The density of PACAP-IR was increased in the superficial laminae (I–II) of the L1, L2, L6, and S1 spinal segments (Vizzard, 2000d). Staining also increased dramatically in a fiber bundle extending ventrally from Lissauer's tract in lamina I along the lateral edge of the dorsal horn to the sacral parasympathetic nucleus in the L6 –S1 spinal segments (lateral collateral pathway of Lissauer). After chronic cystitis, PACAP-IR in cells in the L1, L2, L6, and S1 DRG increased significantly, and the percentage of bladder afferent cells expressing PACAP-IR also increased significantly (70–85%) (Vizzard, 2000d).

19.2.3 PACAP/VIP receptor expression in LUT and modulation with cystitis

19.2.3.1 Urinary bladder

With acute CYP-induced cystitis, PAC1 receptor transcript exhibited a significant decrease in expression in both urothelium and detrusor; however, a significant increase in PAC1 receptor transcript expression in urothelium and detrusor smooth muscle was induced by intermediate and chronic CYP-induced cystitis (Girard et al., 2008).

19.2.3.2 Lumbosacral dorsal root ganglia (DRG)

CYP-induced inflammation of the urinary bladder only affected receptor transcript expression in L6-S1 DRG following acute (4 hr) CYP-induced cystitis. PAC₁ and VPAC₂ receptor transcript expression significantly decreased in both L6 and S1 DRG with acute (4 hr) CYP-induced cystitis. For VPAC₁ receptor transcript expression, a significant increase in expression was demonstrated in the S1 DRG after acute CYP-induced cystitis. No changes in PAC₁, VPAC₁ or VPAC₂ receptor expression in L6 or S1 DRG were demonstrated with intermediate (48 hr) or chronic (8 day) CYP-induced cystitis. PACAP transcript expression significantly increased in the urothelium with intermediate (48 hr) and chronic (8 days) CYP treatment whereas no changes were observed with acute (4 hr) CYP-induced cystitis in either urothelium or detrusor smooth muscle (Girard et al., 2008). Changes in PACAP transcript with CYP-induced cystitis mirrored those observed in the urinary bladder with acute CYP-induced cystitis decreasing and intermediate (48 hr) and chronic (8 day) treatments significantly increasing PACAP transcript expression in both the L6 and S1 DRG (Girard et al., 2008). In a rat CYP-induced cystitis paradigm, intrathecal or intravesical administration of PAC₁

receptor antagonist, PACAP6-38, reduced cystitis-induced bladder hyperreflexia (Braas et al., 2006). These studies demonstrate that PACAP/receptor are modulated by CYP-induced cystitis in tissue-specific ways and that PACAP/receptor signaling plays a role in urinary bladder afferent pathways after urinary bladder inflammation

19.2.4 PACAP in micturition reflexes and modulation after spinal cord injury (SCI)

SCI rostral to the lumbosacral spinal cord alters the coordination between the urinary bladder and external urethral sphincter in many species and results in detrusor sphincter dyssynergia (DSD) that interferes with efficient voiding and results in urinary retention, bladder hypertrophy, increased voiding pressures, increased bladder capacity, and numerous non-voiding contractions (NVCs) during bladder filling (Vizzard, 2006). PACAP is upregulated in micturition reflex pathways after SCI. These studies (Zvara et al., 2006) demonstrate that intrathecal (L6-S1) administration of the PAC₁ receptor antagonist, PACAP6-38 (10 nM), significantly reduced intermicturition interval, threshold and micturition pressures, and number and amplitude of NVCs after SCI (Zvara et al., 2006). In addition, PACAP6-38 increased voiding frequency (i.e., decreased bladder capacity) after SCI (Zvara et al., 2006). In contrast, intravesical administration of PACAP6-38 was without any effect, possibly due to lack of penetration of the PAC₁ antagonist through the urothelium. The presence of PAC₁ receptor transcript in the lumbosacral spinal cord and DRG has been demonstrated (Girard et al., 2008). Thus, intrathecal administration of PACAP6-38 may act at the lumbosacral spinal cord or DRG. The effects of PACAP6-38 after SCI are consistent with PACAP-27 facilitation of micturition in rats (Ishizuka et al., 1995a). PACAP6-38 reduced the number and

amplitude of NVCs after SCI (Zvara et al., 2006). This may be attributed to a reduction in DSD or through an effect on C-fiber bladder afferents (Cheng et al., 1995). An effect of PACAP6-38 on urinary bladder C-fiber afferents is consistent with previous studies that demonstrate that capsaicin depletes PACAP-IR in the LUT (Fahrenkrug and Hannibal, 1998) and that PACAP-IR nerve fibers in the bladder express the vanilloid receptor (Zvarova et al., 2005). PACAP expression is upregulated in micturition pathways after SCI and the present studies demonstrated improved bladder function after intrathecal PACAP antagonist administration. Additional studies addressing the role of PACAP in micturition reflexes suggest that after SCI, PACAP-38 activates spinal circuitry to facilitate the parasympathetic outflow to the urinary bladder and that the elimination of sympathetic pathways enhances this effect (Yoshiyama and de Groat, 2008a; 2008b).

19.2.5 PACAP and VIP expression and effects on major pelvic ganglion (MPG) neurons

Tissue culture experiments modeling neuronal injury have shown PACAP expression regulation in the MPG, the ganglia supplying the majority of autonomic (both sympathetic and parasympathetic) innervation to the bladder, other urogenital organs and components of the lower bowl. While PACAP expression was devoid in acute (4 hour (hr) cultures (Tompkins et al., 2010), both PACAP-IR and PACAP transcript levels increased significantly by day 3 (Girard et al., 2010a). PACAP was preferentially expressed in parasympathetic neurons (Girard et al., 2010a). Transcripts for VPAC1, VPAC2 and PAC1 were present by 4 hr culture (Tompkins et al., 2010), but only VPAC2 transcript levels increased by day 3 (Girard et al., 2010a). Local application of PACAP

or maxadilan, a PAC1-selective agonist, decreased after hyperpolarization and increased neuronal excitability in a subpopulation of neurons (Tompkins et al., 2010). Therefore, PACAP receptor signaling in the MPG may represent another mechanism for neuropeptide-modulated bladder function. Additionally, we have demonstrated VIP expression in the MPG, and local application of VIP results in a decreased after hyperpolarization and increased neuronal excitability in a subpopulation of neurons (Tompkins et al., 2010). However, VIP expression did not increase with prolonged culture (3 days) (Girard et al., 2010a).

19.2.6 PACAP null mice exhibit altered bladder function and somatic sensitivity

PACAP contributions to micturition and somatic sensation were recently studied in PACAP knockout ($PACAP^{-/-}$), littermate heterozygote ($PACAP^{+/-}$) and wildtype (WT) mice using conscious cystometry with continuous intravesical saline or acetic acid (AA; 0.5%) instillation, urination patterns, somatic sensitivity testing of hindpaw and pelvic region with calibrated von Frey filaments and morphological assessments of urinary bladder (May and Vizzard, 2010). $PACAP^{-/-}$ mice exhibit increased bladder mass with fewer but larger urine spots (Fig. 19.2). In $PACAP^{-/-}$ mice, the lamina propria and detrusor smooth muscle are significantly thicker whereas the urothelium is unchanged (Fig. 19.2). $PACAP^{-/-}$ mice exhibit increased bladder capacity, void volume (VV) and longer intercontraction interval (ICI) with significantly increased detrusor contraction duration and large residual volume (May and Vizzard, 2010) (Fig. 19.2). WT mice respond to AA (0.5%) with a reduction in VV and a decreased ICI whereas $PACAP^{+/-}$ and $PACAP^{-/-}$ mice do not respond. $PACAP^{-/-}$ mice are less responsive to somatic

stimulation. PACAP^{+/-} also exhibit bladder dysfunction and somatic and visceral sensory abnormalities but to a lesser degree (May and Vizzard, 2010). PACAP gene disruption contributes to changes in bladder morphology, bladder function and somatic and visceral hypoalgesia (May and Vizzard, 2010).

19.2.7 VIP/receptors and micturition reflex pathways

VIP exerts, species-specific, excitatory or inhibitory actions in neural pathways controlling micturition and these functions may be altered with neural injury, disease or inflammation (Erol et al., 1992; Igawa et al., 1993; Uckert et al., 2002; Hernandez et al., 2006). A number of diverse and conflicting roles for VIP have been demonstrated in the urinary bladder from numerous species. VIP has been shown to relax urinary bladder from human (Uckert et al., 2002) or pig (Hernandez et al., 2006), and contract or produce no effects on urinary bladder from the rat (Erol et al., 1992; Igawa et al., 1993). These contradictory findings might be attributable to species differences (Uckert et al., 2002) and differential VIP receptor distribution (Table 19.1). The majority of VIP in the LUT is located in postganglionic efferent neurons of the pelvic ganglia (Chapple et al., 1992; Smet et al., 1997; Wanigasekara et al., 2003). Surprisingly, VIP had no apparent effects on either bladder tone or EFS-stimulated contractions despite VPAC₂ receptor transcript expression in detrusor (Braas et al., 2006). However, as VIP innervation to the urinary bladder is minimal compared to that for PACAP (Fahrenkrug and Hannibal, 1998), these results may be in keeping with suggestions that PACAP and PAC₁ signaling are more prominent regulators of rat bladder physiology (Braas et al., 2006). These VIP results are consistent with previous studies that demonstrated that VIP application to detrusor

smooth muscle had no effect on spontaneous or carbachol-induced bladder contractions despite facilitation of micturition when VIP was administered intrathecally or intraarterially close to the rat bladder (Igawa et al., 1993). Recent studies (Studený et al., 2008) using VIP^{-/-} mice reveal that VIP^{-/-} mice exhibit increased bladder mass and fewer but larger urine spots on filter paper. VIP^{-/-} mice exhibit increased void volumes and shorter intercontraction intervals with continuous intravesical infusion of saline (Studený et al., 2008). No differences in transepithelial resistance or water permeability were demonstrated between VIP^{-/-} and WT mice (Studený et al., 2008). With the induction of bladder inflammation by acute administration of CYP, an exaggerated or prolonged bladder hyperreflexia was demonstrated in VIP^{-/-} mice (Studený et al., 2008). The changes in bladder hyperreflexia may reflect increased expression of neurotrophins and/or or proinflammatory cytokines in the urinary bladder (Studený et al., 2008).

19.2.8 PACAP-mediated ATP release from cultured, rat urothelium

Earlier studies have indicated that the urothelium releases ATP in response to various stimuli (Birder, 2006). In addition, it has been suggested that adenosine triphosphate (ATP) released from the serosal surface of the urothelium during bladder filling stimulates receptors on suburothelial sensory nerve fibers and contributes to bladder filling sensation (Cockayne et al., 2000). ATP in the cell cytoplasm can be released extracellularly by several mechanisms including exocytosis of ATP-containing vesicles (Bodin and Burnstock, 2001; Novak, 2003). PACAP27, PACAP38 and VIP application evoked ATP release from rat urothelial cell cultures; however, ATP release was greatest with PACAP27 treatment and significantly blocked by the PAC₁ receptor selective

antagonist, M65 (Girard et al., 2008). Current research supports the suggestion that PACAP and PAC₁ signaling are regulators of bladder physiology at the level of the urinary bladder and specifically, the urothelium (Girard et al., 2008).

19.3 Tachykinins and calcitonin-gene related peptide (CGRP) in micturition reflex pathways

A variety of neuropeptides are present in the somata and processes of urogenital DRG cells including urinary bladder and urethra with CGRP and Substance P (SP) being the most widely distributed (Keast, 1992). Tachykinins (SP, neurokinin A, neurokinin B) and CRGP are present in the LUT and act on neurokinin (NK)1, NK2, NK3 or CGRP receptors, respectively (Andersson, 2002; Canda et al., 2006). NK1 and NK2 receptors have been reported in the detrusor smooth muscle whereas NK2 receptors are present in the urothelium (Ishizuka et al., 1995b; Birdier, 2010). In broad terms, the sensory functions of the tachykinins include regulation of micturition threshold, activation of cardiovascular reflexes and perception of pain from the urinary bladder (Maggi et al., 1995; Gu et al., 2000; Andersson, 2002). Efferent functions of the tachykinins include regulation of local muscle cell activity, nerve excitability, plasma extravasation and blood flow (Andersson, 2002). Involvement of tachykinins located in supraspinal sites on micturition function has also been demonstrated (Gu et al., 2000).

19.3.1 Tachykinins and CGRP in micturition reflex pathways and regulation with inflammation, injury or disease

Expression of tachykinins and associated receptors in the LUT under basal conditions and alterations in expression has been reported in animal models of bladder inflammation

and in the clinical syndrome of IC/PBS. Recent studies have demonstrated alterations in SP-immunoreactivity (IR) (Pang et al., 1995) and NK1 mRNA (Marchand et al., 1998) in bladder biopsies from patients with IC/PBS (Johansson and Fall, 1994; Ho et al., 1997; Johansson et al., 1997). In addition, a study involving an acute rat model of urinary bladder inflammation (48 hr following intravesical mustard oil treatment) has demonstrated significant increases in CGRP- and SP-IR in rostral (L1, L2 DRG) and caudal (L6, S1 DRG) bladder afferent neurons (Callsen-Cencic and Mense, 1997). Furthermore, it has been demonstrated (Lecci et al., 1994) that intrathecal injection of SP antagonists reduce CYP-induced bladder hyperreflexia. Although numerous studies have demonstrated changes in CGRP and SP expression in sensory neurons following nerve injury (Hokfelt et al., 1994) or peripheral inflammatory states (Kataeva et al., 1994; Luber-Narod et al., 1997; Traub et al., 1999; Hutchins et al., 2000), there are only a limited number of studies that have examined alterations in CGRP or SP expression following the induction of acute urinary bladder inflammation (Callsen-Cencic and Mense, 1997; Luber-Narod et al., 1997). Increases in CGRP- and SP-IR in bladder afferent neurons in the lumbosacral DRG 48 hr following the induction of cystitis with intravesical mustard-oil have been demonstrated (Callsen-Cencic and Mense, 1997).

19.3.2 SP and CGRP plasticity in LUT pathways with CYP-induced cystitis

Additional plasticity of CGRP and SP expression in LUT pathways has been demonstrated in the CYP model of bladder inflammation in rats (Vizzard, 2001). In control rats, CGRP- or SP-IR was expressed in fibers in the superficial dorsal horn in all segmental levels examined (L4-S1). Bladder afferent cells in the dorsal root ganglia

(DRG; L6, S1) from control animals also exhibited CGRP- (41–55%) or SP-IR (2–3%). Following chronic, CYP-induced cystitis, CGRP- and SP-IR were dramatically increased in spinal segments and DRG (L6, S1) involved in micturition reflexes. The density of CGRP- and SP-IR was increased in the superficial laminae (I-II) and lateral collateral pathway of the L6 and S1 spinal segments. Following chronic cystitis, CGRP- and SP-IR in cells in the L6 and S1 DRG significantly increased and the percentage of bladder afferent cells expressing CGRP- (76%) or SP-IR (11–18%) also significantly increased (Vizzard, 2001).

The functional significance of an upregulation of CGRP or SP in bladder pathways following CYP-induced cystitis is not known but changes in neuropeptide expression and presumably release at both central and/or peripheral projections of afferent pathways are possible. Several reports have suggested that neuropeptide-containing, capsaicin-sensitive bladder afferents may mediate urinary bladder hyperreflexia (Maggi, 1991; Giuliani et al., 1993a; Giuliani et al., 1993b; Ahluwalia et al., 1994; Ahluwalia et al., 1998). It has also been shown that intrathecal SP facilitates normal micturition and SP antagonists delivered intrathecally depress normal micturition indicating that the peptide may be involved as an excitatory transmitter in several types of bladder reflexes in the rat (Mersdorf et al., 1992; Lecci et al., 1993). CYP treatment in the rat induces cystitis that is characterized by increased frequency of voiding in awake rats and urinary bladder hyperreflexia in anesthetized rats (Maggi et al., 1992; Lecci et al., 1994; Lanteri-Minet et al., 1995). Furthermore, it has been demonstrated (Lecci et al., 1994) that intrathecal injection of SP antagonists reduce CYP-induced bladder

hyperreflexia. Intrathecal administration of neurokinin receptor (NK)1 antagonists (RP 67580 and CP 96345) increase bladder capacity in normal conscious rats with no changes in voiding pressure whereas NK2 receptor antagonists were ineffective (Lecci et al., 1994). Bladder hyperreflexia induced by capsaicin was reduced by an NK2 antagonist (SR 48965) with no effects on normal micturition (Lecci et al., 1994). Exogenous CGRP application or CRGP release from primary afferent nerves relaxes smooth muscle and produces relaxation with bladder effects being more prominent in guinea pig and dog compared to rat and human (Maggi, 1992; Andersson, 1993). In addition to pharmacological demonstration of involvement of tachykinins/receptors systems in the LUT, studies with the preprotachykinin A (that encodes both SP and neurokinin A) null mouse, have demonstrated involvement of tachykinins in the response to chemical irritation of the LUT as well as to regulation of normal micturition activity (Kiss et al., 2001).

Recent studies with cizolirtine citrate, an inhibitor of CGRP and SP release at the spinal cord level, showed a significant reduction in the total number of voids per 24 hr in patients with urinary incontinence secondary to OAB (Zat'ura et al., 2010). Further, cizolirtine citrate resulted in improvement in urinary incontinence and urgency in symptomatic outpatients with OAB and/or urodynamic diagnosis of detrusor overactivity (Martinez-Garcia et al., 2008). Therefore, it is possible that increased expression of CGRP- or SP-IR in bladder afferent cells and central and peripheral projections could contribute to this hyperreflexia. Changes in neuropeptide expression and release at central terminals could further result in a remodeling of spinal cord circuitry controlling

micturition (Lecci et al., 1994). This remodeling may include changes: (1) in the synaptic organization of spinal micturition reflexes; (2) in the neurochemical coding of specific neuronal elements (primary afferent neurons, interneurons) and (3) in the organization of ascending and descending projections to spinal reflexes. Further, recent studies also demonstrate that SP released from nerve fibers or urothelial cells can act on urothelial receptors to release nitric oxide (Birder, 2006; Birder, 2010). In response to stimulation of receptors on urothelial cells, SP and neurokinin A can be released from urothelial cells. Thus, neural as well as non-neural tachykinins may contribute to LUT pathways. Non-neural involvement of neuropeptides in the LUT has been recently addressed (Birder, 2006; Birder, 2010).

The exact role(s) of tachykinins in urethral physiology is not known but tachykinins induce urethral contraction in many species including humans (Maggi et al., 1988; Maggi, 1992; Palea et al., 1996; Parlani et al., 1996; Canda et al., 2006; 2008). In patients with genitourinary prolapse with concomitant urinary incontinence, the density of SP-immunoreactive nerves in the perineal muscles was significantly decreased compared to a continent group suggestive of a role for tachykinins in urinary continence mechanisms (Busacchi et al., 2004). Additional research focused on the role of tachykinins in urethral physiology is necessary.

19.4 Role of Neurotrophic Factors in Neuropeptide Expression in Micturition Pathways after CYP-induced Cystitis

Altered bladder neurotrophic factor content may underlie neurochemical (Vizzard and de Groat, 1996; Vizzard, 2000d; Vizzard, 2000c; Vizzard, 2000a; Vizzard, 2001)

changes in bladder afferent neurons after cystitis. The occurrence of trophic interactions between nerve cells and target tissues is clearly demonstrated during embryonic and postnatal development (Lindsay et al., 1990; Oppenheim et al., 1991; Lapchak et al., 1992; Vantini and Skaper, 1992). Recent experiments have demonstrated the influence of interactions between target organ and neurons in adult animals (Steers and de Groat, 1988; Steers et al., 1991a; Steers et al., 1991b; Tuttle and Steers, 1992, Tuttle et al., 1994; Vizzard, 2000b; Dupont et al., 2001). NGF is expressed under normal conditions in the urinary bladder, and part of its function is likely maintenance of sensory afferent fibers (Chao and Hempstead, 1995; Chuang et al., 2001). We have demonstrated that chronic CYP-induced cystitis alters NGF expression and other neurotrophic factors in the bladder (Vizzard, 2000b). The role(s) of NGF in micturition reflexes and sensation have been evaluated using a variety of addition and subtraction techniques (Dmitrieva and McMahon, 1996; Clemow et al., 1998; Yoshimura et al., 2006). Importantly, exogenous NGF application to the rat bladder detrusor through an osmotic pump reduced bladder capacity and increased expression of CGRP in the lumbosacral spinal cord (Zvara and Vizzard, 2007)(Fig. 19.3).

Recent studies involving a novel NGF overexpressing (OE) mouse model (Cheppudira et al., 2008; Schnegelsberg et al., 2010) are being used to further define NGF-mediated changes in LUT structure and function and effects on neuropeptide/receptor systems (Fig. 19.4). The urinary bladder in NGF OE mice exhibits marked hyperinnervation. To characterize the sub-populations of neurons contributing to

the generalized hyperinnervation observed in NGF-OE transgenic mice, we have performed whole-mount immunostaining of bladder urothelium using a panel of neuronal markers. Using the pan-neuronal marker PGP 9.5, a marked increase in total nerve fiber density was seen in the urinary bladders of transgenic mice compared to WT controls (Fig. 19.4), consistent with the histology stains. This dense network was composed of CGRP-positive (Fig. 19.4) and substance P-positive (Fig. 19.4) unmyelinated C-fiber sensory afferents, NF 200-positive myelinated sensory afferents and TH-positive, post-ganglionic sympathetic nerve fibers (Schnegelsberg et al., 2010). The increased nerve fiber density observed in transgenic mouse urinary bladders was evident in both the neck and the dome region of the urinary bladder. However, we observed a higher innervation density within the urinary bladder neck region compared to the dome in both WT and transgenic mice. With CGRP immunostaining, we also observed CGRP-positive ganglia (5-9 CGRP-positive cells per ganglia) interspersed among CGRP-positive nerve fibers within the suburothelial plexus in transgenic mice. These CGRP-positive ganglia were not observed in whole-mount preparations of WT urinary bladder (Schnegelsberg et al., 2010). Studies are currently underway to examine effects of neuropeptide/receptor blockade on bladder function in NGF OE mice that exhibit bladder hyperreflexia with the presence of NVCs (Schnegelsberg et al., 2010).

Recent studies also demonstrate changes in PACAP/VIP and receptor expression in micturition pathways in NGF-OE mice (Girard et al., 2010b). Results demonstrate upregulation of PAC1 receptor transcript and PAC1-immunoreactivity in urothelium of

NGF-OE mice whereas PACAP transcript and PACAP-immunoreactivity were decreased in urothelium of NGF-OE mice (Girard et al., 2010b). In contrast, VPAC1 receptor transcript was decreased in both urothelium and detrusor smooth muscle of NGF-OE mice (Girard et al., 2010b). VIP transcript expression and immunostaining was not altered in urinary bladder of NGF-OE mice (Girard et al., 2010b). Changes in PACAP, VIP and associated receptors transcripts and protein expression in micturition pathways resemble some, but not all, changes observed after induction of urinary bladder inflammation known to involve NGF production.

19.5 Bradykinin/receptors system in micturition reflex pathways

The bradykinin/receptors system plays an important role in normal micturition reflexes as well as in pathology (Lecci et al., 1995; Belichard et al., 1999; Lecci et al., 1999; Meini et al., 2000; Chopra et al., 2005; Fabiyi and Brading, 2006). Kinins are small peptides (8-10 amino acids) produced in plasma or in tissues at the sites of inflammation or tissue damage (Bhoola et al., 1992). Protease activation at the site of inflammation or tissue damage cleaves tissue/plasma kininogen precursors to release the nonapeptide, bradykinin (Dray and Perkins, 1993). The biological effects of bradykinin are mediated by two different receptors, B1 and B2 with the bradykinin B2 receptor being constitutively expressed in various cell types and the B1 receptor being expressed *de novo* after inflammatory stimuli or tissue injury (Marceau et al., 1998; Lecci et al., 1999; Chopra et al., 2005). Recent studies (Chopra et al., 2005) have demonstrated B2 receptor expression in the detrusor smooth muscle and urothelium (apical and basal cells) consistent with reported bradykinin-evoked contractility of detrusor smooth muscle. It

has been recently demonstrated that bradykinin-induced facilitation of micturition reflexes may be due to an increase in purinergic (P2) responsiveness (Chopra et al., 2005). Further, stimulation of the B2 receptor in cultured rat urothelial cells resulted in ATP release (Chopra et al., 2005). ATP release was reduced by the selective B2 receptor antagonist Hoe-140 and suggests that bradykinin may elicit bladder hyperreflexia indirectly through the release of ATP from the urothelium (Chopra et al., 2005). Direct effects of bradykinin on pelvic afferent nerves that evoke bladder hyperreflexia have also been demonstrated (Lecci et al., 1995). Thus, direct (neuronal) (Lecci et al., 1995) and indirect (urothelial secretion of ATP) (Chopra et al., 2005) actions of bradykinin on the LUT are likely to affect micturition reflexes.

19.5.1 Bradykinin/receptors in cystitis

The role of the bradykinin/receptor system in the LUT varies under control or pathological states. It is largely agreed that B1 receptor expression is undetectable in control urinary bladder (Ahluwalia and Perretti, 1999; Chopra et al., 2005). Application of the selective B1 receptor agonist, des-Arg⁹-bradykinin exerts little or no effect on $[Ca^{2+}]_i$ or ATP release from cultured rat urothelial cells consistent with no or very low constitutive B1 receptor expression (Chopra et al., 2005). CYP-induced cystitis significantly upregulates B1 receptor expression in the detrusor smooth muscle and urothelium with acute (24 hr) cystitis (Chopra et al., 2005). In cultured rat urothelial cells obtained from CYP (24 hr)-treated rats, the B1 receptor agonist, des-Arg⁹-bradykinin, evoked release of ATP and elevated calcium levels (Chopra et al., 2005). B1 receptor expression is also upregulated in bladder biopsies from patients with IC/PBS (Ruggieri et al., 1997). Cystometry performed on CYP (24 hr)-treated rats demonstrated that the B1

receptor antagonist, des-Arg¹⁰-Hoe-140, significantly reduced the frequency of non-voiding bladder contractions (Chopra et al., 2005). In contrast, the B2 receptor antagonist, Hoe-140, decreased voiding frequency in CYP (24 hr)-treated rats (Chopra et al., 2005). It has been suggested that one explanation for these findings is the existence of B1 sensitive mechanisms/or afferent pathways underlying the emergence of non-voiding contractions and B2 sensitive mechanisms underlying voiding contractions (Chopra et al., 2005). Additional studies are needed to address this possibility. Interestingly, chronic CYP-induced cystitis (8 days) resulted in a return to baseline of B1 receptor expression in the detrusor smooth muscle whereas expression in the urothelium was similar to that observed with acute (24 hr) CYP-induced cystitis (Chopra et al., 2005). Bradykinin/receptors systems may exert tissue-specific and inflammatory duration-dependent effects on LUT reflexes.

19.6 Endothelin/receptors system in LUT pathways

Endothelin-1 and endothelin receptors, ET_A and ET_B, contribute to LUT function under normal and pathological conditions (Ukai et al., 2006; Ogawa et al., 2008; Ukai et al., 2008). In the LUT, endothelin-1 facilitates detrusor smooth muscle contraction in various species including rabbit, rat, dogs and humans and stimulates proliferation of the prostate and urinary bladder (Maggi et al., 1989; Garcia-Pascual et al., 1990; Persson et al., 1992; Langenstroer et al., 1997; Ogawa et al., 2004). Studies have demonstrated differential endothelin-1 contractile mechanisms depending on species (Persson et al., 1992). Differential endothelin receptor density has been revealed in the LUT with the greatest density of ET_A receptors being present in the ureter (Latifpour et al., 1995; Saenz

de Tejada et al., 1992; Afiatpour et al., 2003). In humans and other animals, ET_A expression is more dominant than ET_B in the bladder dome compared to the bladder base or urethra, which exhibits equal ET_A and ET_B receptor expression (Saenz de Tejada et al., 1992; Latifpour et al., 1995; Afiatpour et al., 2003). Endothelin expression has been detected in detrusor smooth muscle, urothelium, and vascular endothelium (Saenz de Tejada et al., 1992; Latifpour et al., 1995; Afiatpour et al., 2003).

19.6.1 Endothelin/receptor system in bladder outlet obstruction and spinal cord transection

In animal models of BOO, modulation of endothelin-1 and ET_A and ET_B expression has been demonstrated in the LUT and studies are researching the endothelin/receptor system as a potential therapeutic target for the treatment of bladder overactivity secondary to BOO (Khan et al., 1999; Ukai et al., 2008). BOO in rabbits is associated with increased expression of ET_A, ET_B and endothelin-1 in detrusor smooth muscle (Khan et al., 1999; Ukai et al., 2008). The ET_A selective receptor antagonist, YM598, dose-dependently reduced the frequency of premicturition bladder contractions in BOO rats without effects on other voiding parameters (Ukai et al., 2008). Further, inhibition of the endothelin-converting enzyme, which metabolizes big endothelin to endothelin-1, decreased bladder overactivity in BOO rats (Schroder et al., 2004). Additional beneficial effects of repeated administration of endothelin-converting enzyme inhibitor in BOO rats included normalization of: bladder weight, micturition pressures and voiding durations (Schroder et al., 2004). Suppression of ET_A receptors by intravenous infusion of ABT-627 in rats with complete spinal cord transection (thoracic

8-9) significantly decreased the amplitude and number of non-voiding bladder contractions without changing bladder pressures, void volumes or voiding efficiency (Ogawa et al., 2008). No effects on bladder function were observed when rats with spinal cord transection were similarly treated with an ET_B receptor antagonist, A-192621 (Ogawa et al., 2008). Thus, endothelin and ET_A receptors may be a novel therapeutic target to ameliorate the effects of bladder overactivity associated with BOO.

Additional studies are necessary to address the mechanisms by which YM598, an endothelin ETA receptor antagonist, suppresses the bladder overactivity observed in BOO rats; potentiation of postsynaptic norepinephrine effects and endothelin-1 increase of interleukin-(IL) 6 have been suggested (Han et al., 2009). Interestingly, recent studies have demonstrated that endothelin-converting enzyme 1 promotes the recycling and re-sensitization of NK1 receptors and re-sensitization of the pro-inflammatory effects of SP (Cattaruzza et al., 2009). Thus, reductions in bladder overactivity secondary to BOO observed with endothelin-converting enzyme inhibition (described above) may, in part, be due to amelioration of the pro-inflammatory effects of SP (Cattaruzza et al., 2009).

19.6.2 Endothelin/receptor systems in the urethra

There is very limited information concerning the distribution and function of endothelin/receptor systems in the urethra. ET receptors have been demonstrated in the urethral smooth muscle in several species (Latifpour et al., 1995; Afiatpour et al., 2003). ET_A-mediated smooth muscle contractions of the urethra in the rabbit have been demonstrated (Wada et al., 2000).

19.7 Perspectives

A large body of research supports a major role for neuropeptides in LUT function in health and disease thereby identifying neuropeptide/receptor systems as potential novel therapeutic targets for the treatment of LUT disorders (Fig. 19.1). In animal models, pharmacological and/or genetic manipulation of SP, CGRP, VIP, PACAP, bradykinin or endothelin function affects LUT function. Further, in animal models of LUT dysfunction or in clinical assessments, manipulation of neuropeptide/receptor systems improves void function. Furthermore, the field has become increasingly complex, with neuronal plasticity exhibited in pathophysiological situations where changes in neuropeptide expression patterns and receptor density are regularly observed. The study of neuropeptides in animal models has additionally revealed physiological and pathophysiological roles that in turn have led to the ongoing development of new drugs, through utilization predominantly of antagonist activities or blockade of release.

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Table Legends

Table 19.1: Summary of VIP/PACAP receptor isoforms and tissue distribution in the lower urinary tract

Figure Legends

Figure 19.1: Neuropeptide/receptor systems expression in micturition reflex pathways emphasizing bladder afferent and urothelium participation. A.

Diagram of afferent innervation of the urinary bladder. Numerous neuropeptide/receptor systems have been identified in bladder afferent (i.e., sensory) pathways with contributions to normal lower urinary tract function as well as that after neural injury, disease or inflammation. **B.** Potential model of possible reciprocal neuropeptide/receptor interactions among bladder afferent and efferent nerves (not shown), urothelial cells, myofibroblasts located in the suburothelium and detrusor smooth muscle that underlie physiological bladder reflex function as well as pathophysiology in bladder disease. Receptor activation and channel stimulation on urothelial cells can elicit secretion of chemical mediators that can affect adjacent tissues including bladder afferent nerves in the suburothelial plexus, myofibroblasts and detrusor smooth muscle. Urothelial cells can also be responsive to neurotransmitters released from bladder nerves and other cell types including inflammatory cells. Abbreviations: ATP, adenosine triphosphate; TrkA, receptor tyrosine kinase A; p75^{NTR}, low affinity neurotrophin receptor; PG, prostaglandin; NO, nitric oxide; NGF, nerve growth factor; NP, neuropeptides; Ach, acetylcholine; PAC₁, pituitary adenylate cyclase-activating polypeptide (PACAP) selective receptor; VPAC₂, receptor with equal and high affinity for vasoactive intestinal polypeptide and PACAP; B₁, bradykinin receptor 1; B₂, bradykinin receptor 2; ET_A, endothelin receptor A; ET_B, endothelin

receptor B; NK2, neurokinin receptor 2; NK1, neurokinin receptor 2; NK3, neurokinin receptor 3; CGRP1, calcitonin gene-related receptor. See text for additional details. Figure modified from (Arms et al., 2009).

Figure 19.2: PACAP contributions to bladder morphology and function. A-C. Histological analyses of urinary bladders and bladder function among PACAP^{-/-}, PACAP^{+/-} and WT mice. Representative haematoxylin and eosin stained cryostat bladder sections (15 µm) from WT (A) and PACAP^{-/-} (B) mice that demonstrate significantly (*, p≤ 0.01) increased thickness of lamina propria (LP) and detrusor smooth muscle (SM) supported by morphometric analyses (C). D-E. Summary figures of the intercontraction interval (ICI, seconds, s; D) and residual volume (RV, µl; E) using conscious cystometry in conscious, unrestrained WT, PACAP^{-/-} and PACAP^{+/-} mice with continuous infusion of saline or AA (0.5%). D. ICI was significantly (p≤ 0.01) longer in PACAP^{-/-} compared to WT mice with instillation of saline and AA. No changes in ICI were detected with intravesical instillation of AA in PACAP^{-/-} and PACAP^{+/-} mice compared to saline instillation. ICI was significantly (p≤ 0.01) greater in PACAP^{-/-} and PACAP^{+/-} mice with AA instillation compared to WT. E. RV was significantly (p≤ 0.01) increased in PACAP^{+/-} and PACAP^{-/-} mice compared to WT. Values represent mean ± S.E.M. for n = 7-10 animals in each group. U, urothelium; WT, wildtype. Calibration bar represents 120 µm in A, B. Figure modified from (May and Vizzard, 2010).

Figure 19.3: CGRP spinal cord expression with exogenous NGF treatment.

CGRP-IR increases in lumbosacral spinal cord with exogenous NGF treatment.

CGRP-IR in the L6 (A-B) spinal segment in control (A) and NGF-treated (B)

rats. A. Fluorescence photographs showing CGRP-IR in the L6 (A) spinal

segment of control (saline) + bladder distention. B. Fluorescence photographs

showing CGRP-IR in the L6 (B) spinal segment with NGF treatment + bladder

distention. Increased density of CGRP-IR was observed in the medial (MDH) to

lateral (LDH) extent of the superficial laminae (I-II) of the dorsal horn (DH) with

NGF treatment in L6 (C) segments. Summary bar graph of CGRP optical density

(O.D.) as measured in specific regions of the L6 spinal cord (C). Calibration bar

represents 125 μm . SPN, sacral parasympathetic nucleus; DCM, dorsal

commissure; LCP, lateral collateral pathway. *, $p \leq 0.01$. Figure modified from

(Zvara and Vizzard, 2007).

Figure 19.4: Neuronal hyperinnervation in the urinary bladders of NGF-

overexpressing (OE) transgenic mice. Representative fluorescence images of

PGP 9.5 (A, B), CGRP (C, D), SP (E, F), immunoreactivity in the bladder neck

region in urothelial whole-mount preparations of urinary bladders from 8-10

week-old female WT (A, C, E) and NGF-OE transgenic (B, D, F) mice from F0

line 23 ($n = 3-5$). Scale bars: A-F, 50 μm . Protein Gene Product (PGP 9.5),

Calcitonin Gene Related Protein (CGRP), Substance P (SP). Figure modified

from (Schnegelsberg et al., 2010).

Figures

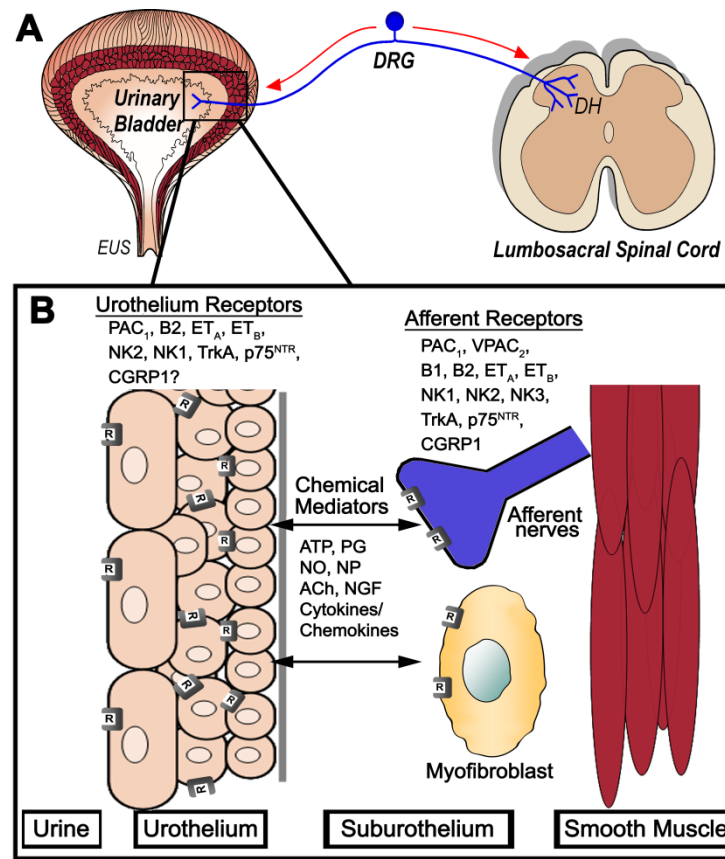


Figure 1

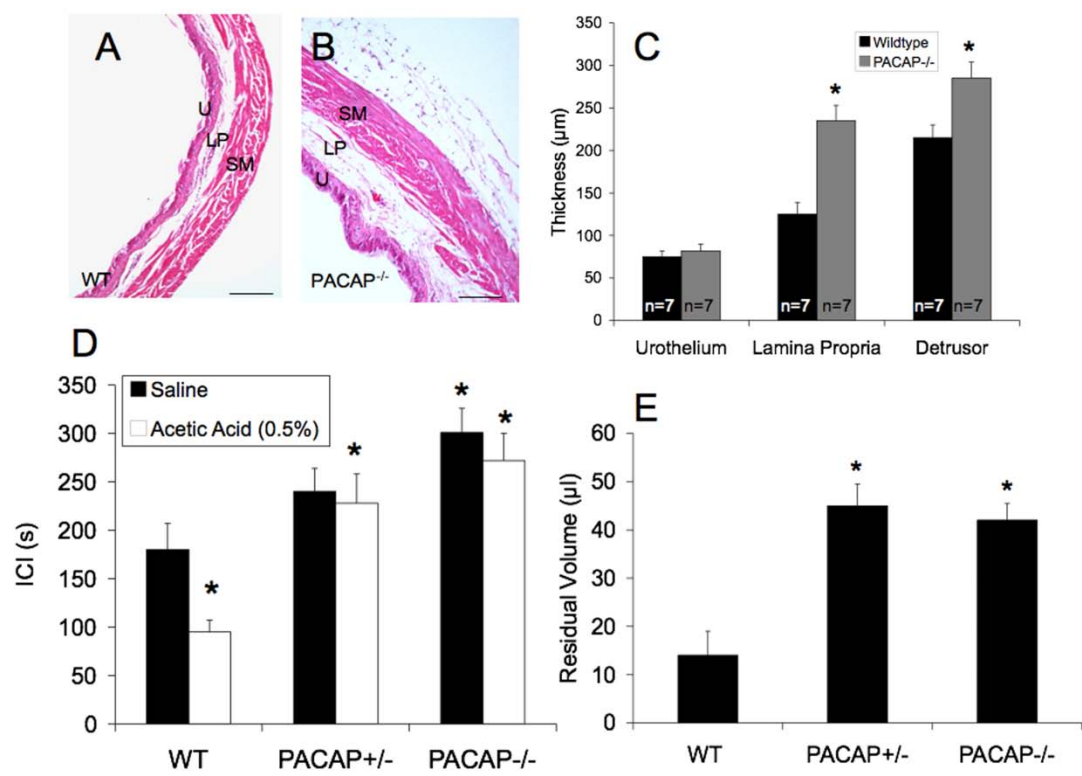


Figure 2

Figure 2

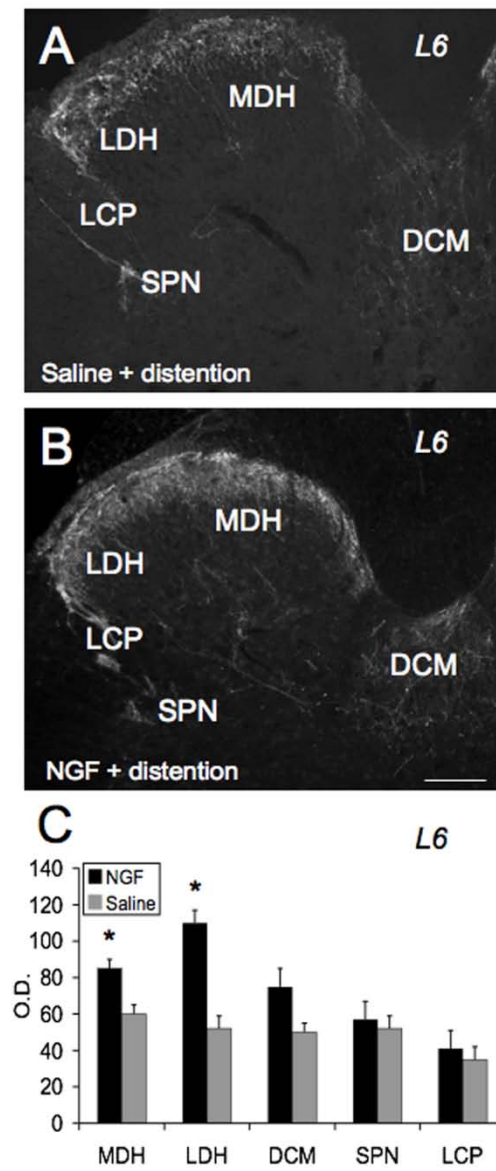


Figure 3

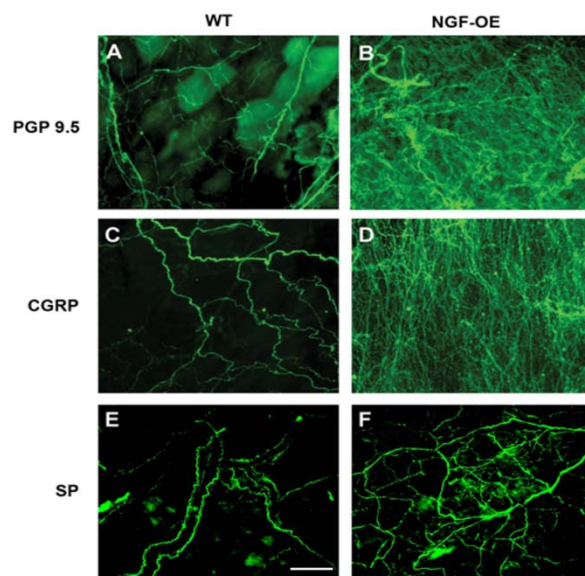


Figure 4